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**THE METABOLIC RESPONSE OF HUHU LARVAE,
PRIONOPLUS RETICULARIS (COLEOPTERA :
CERAMBYCIDAE) TO STRESS FROM ELEVATED
TEMPERATURE AND REDUCED OXYGEN**

A thesis submitted in partial fulfilment
of the requirements for the degree of
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THE UNIVERSITY OF
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ABSTRACT

Huhu, *Prionoplus reticularis* White (Coleoptera: Cerambycidae), are native New Zealand borers of quarantine importance. Huhu do not attack living trees, although their eggs can be laid on freshly-felled timber and larvae may be found in older export timber. To control insect pests, many logs from New Zealand are currently fumigated with methyl bromide (MBr), which has led to research to find alternatives for this ozone-depleting chemical. Treatments using heat and controlled atmospheres require a large amount of insect mortality data, often collected on a trial and error basis, which is time consuming and expensive. Insect physiological responses to treatment stress may complement mortality studies. These physiological studies on huhu sought to determine how huhu larvae respond to stresses caused by elevated temperature and low oxygen atmospheres. The purpose was to identify metabolic responses in huhu that could assist with development of commodity, insect disinfection strategies.

An artificial diet for huhu larvae was developed and tested for rearing uniform insects for physiological studies. Larvae were reared at 20 and 25°C and two day-lengths to identify suitable rearing conditions. The artificial diet was suitable for rearing huhu larvae at 20°C with either day-length. Rearing at 25°C shortened development time initially, but ultimately, larval weight decreased and many died.

Huhu larvae had standard and active metabolic rates at 20°C of 1.66 and 4.42 $\mu\text{mol.kg}^{-1}.\text{s}^{-1}$, respectively as measured by manometric compensatory respirometry. Larval metabolic rate increased 1.72 times in response to a 10°C increase in temperature, over the range of 20 to 42°C. At temperatures greater than 35°C, larval movement ceased, consistent with stress responses reported for other insects.

An automated gas analysis system was constructed and calibrated, which redirected the focus of planned research using the manometric system. SMR for huhu at 20-40°C was lower than predicted values, but within the range of non-flying adult coleopterans and had a mass exponent between 0.62 and 0.67. The respiratory quotient (RQ) increased with temperature, suggesting a shift from metabolising fat to include other substrates. Activity increased larval metabolism but RQ was

independent of activity at 20°C. Temperature sensitivity was similar when measured by gas analysis or manometrically.

Huhu oxygen consumption (\dot{M}_{O_2}) and carbon dioxide production (\dot{M}_{CO_2}) were measured with the automated gas analysis system. When larvae were exposed to extreme constant temperatures, 35-45°C, larval \dot{M}_{O_2} increased until a critical temperature (42/43°C) resulted in falling \dot{M}_{O_2} , which was exacerbated by time. \dot{M}_{CO_2} reached a thermal maximum, suggesting the elimination of carbon dioxide may be a limiting factor. These changes in metabolism were consistent with mortality studies on huhu exposed to heat. Larval \dot{M}_{CO_2} was reduced in 1.8% oxygen and movement ceased, suggesting huhu use metabolic depression to survive hypoxia. Recovery from anoxia increased \dot{M}_{O_2} as larvae repaid an oxygen debt.

Huhu haemolymph pH and lactate levels were measured after exposure to stress. Extreme temperature, reduced oxygen atmospheres and their combinations increased anaerobiosis, indicated by increased haemolymph acidosis and lactate. The metabolic response depended on the severity of the atmosphere-temperature treatment and exposure time.

This study has successfully measured the metabolism of a larval cerambycid, and added valuable knowledge to the relative paucity of studies in this subject area. In response to stress, huhu larvae can utilise anaerobic pathways leading to severe acidosis with large amounts of lactate, in contrast to much of the published literature. The respiratory responses and increased anaerobic respiration found in this study, when an insect is exposed to stress, will assist the development of commodity disinfection protocols and will contribute to a reduction the use of MBr.

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GENERAL INTRODUCTION

Huhu biology

Huhu, *Prionoplus reticularis* White (Coleoptera: Cerambycidae), are native New Zealand long-horn borers, originally found in lowland podocarp timber but now more common in introduced plantation species such as *Pinus radiata* D. Don (Edwards 1961b). Huhu do not attack living trees although they can infest the dead parts of living trees. This type of damage often occurs in *Cupressus* and *Thuja* but is uncommon in *P. radiata*. Successful colonization appears to be related to the cessation of active sap secretion. Huhu are primary colonizers preferring sound timber. They are so efficient at turning logs into frass, which admits water and leads to secondary colonisers, that it is commonly thought they inhabit rotten logs (Edwards 1959).

In the field, most researchers estimate the life cycle of huhu on *P. radiata* to be between three and five years (Morgan 1960, Edwards 1961a, Hosking 1978). Females oviposit about 250 to 350 eggs (Figure 1.1) on fallen or cut logs, stumps, green sawn timber and the dead parts of live trees. The eggs usually hatch after 16 to 25 days, although hatching from a single batch can sometimes be spread over 2 weeks (Morgan 1960). The egg stage is the most susceptible to desiccation resulting in up to 50% egg mortality, even after the larva has pierced the chorion (Edwards 1961a). The neonate larva makes a short establishment gallery perpendicular to the surface of the bark extending about 2 mm into the wood (Edwards 1961a). Further larval tunnelling and feeding is then typically along the axis of the grain and involves the formation of galleries where moulting takes place (Figures 1.1 and 1.2). The pattern of tunnelling and associated larval behaviour depend upon the size of the log, the state of decay and the degree of previous attack (Edwards 1961a).

Morgan (1960) demonstrated huhu have a preference for timber with greater than 40% moisture, and they can not develop in timber with less than 25% moisture. Huhu larvae are almost always found in shaded timber, which is likely to be consistent with larval moisture requirements (personal observation). Interestingly, huhu larvae can survive for up to 2.25 y in the laboratory without food and then

resume feeding again (Edwards 1961a). The ability to survive extended periods of starvation probably assists the larvae to endure adverse conditions such as dry timber. The larval period extends over at least two to three years, and includes an over-wintering, non-feeding final instar. The number of larval instars is unknown. The pupal chamber is constructed by late instar larvae prior to entry into a pre-pupal stage lasting 10-15 d (Figure 1.2) After a 25-day pupation period, adult beetles (Figure 1.3) exit the logs to mate. Maori, the indigenous people of New Zealand, were familiar with the different life-stages of *P. reticularis* and had a separate name for actively feeding larvae, pre-pupae (considered a great delicacy), pupae and adults. The general Maori name for this insect is huhu and has been adopted as its common name.

Figure 1.1 Huhu eggs laid between the bark and sap wood, shown here still attached to the bark after removal (left, eggs are about 3 mm). Huhu larva, weighing 2.5 g (right, scale = 1 cm).

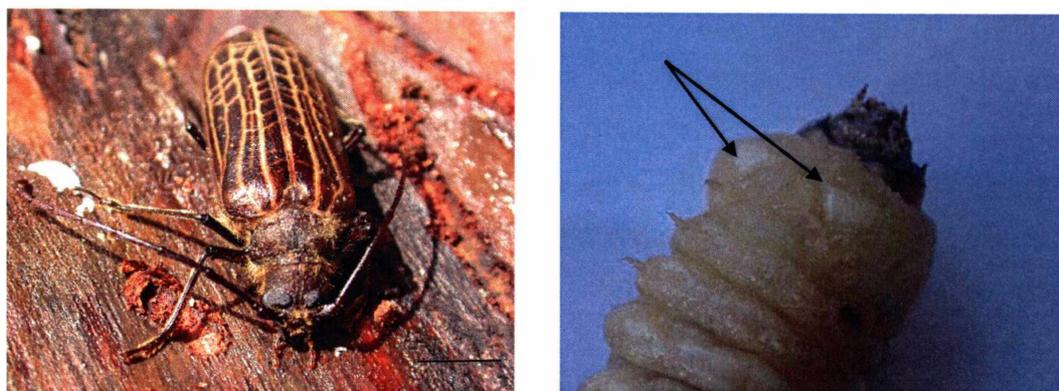


Figure 1.2 Frass-filled tunnels associated with attack from mature larvae. A pupa within its chamber is arrowed (left). The same female pupa within its chamber (right, scale = 1 cm).



Huhu larvae can be distinguished from other cerambycids using the keys of Duffy (1963) or Dumbleton (1957). However, identification of huhu larvae can be achieved using the following characteristics; possession of a combination of pointed mandibles, legs and carinae on the frons of larvae (John Bain, Forest Research Institute, personal communication 1995). Furthermore, two white spots (Figure 1.3) on the ventral surface of larger larvae provide a reliable characteristic for field identification.

Figure 1.3. An adult female huhu beetle (left, scale = 1 cm) and the anterior ventral aspect of a huhu larvae (right) showing the characteristic white spots (arrowed)



Timber disinfestation

Huhu eggs can be laid on freshly felled timber or on cut logs awaiting export, leading to their classification as an economic pest of quarantine importance. Larvae can be found in older export timber. To control huhu and other insect pests, New Zealand logs exported to the USA and Asian markets are currently fumigated with methyl bromide (Anonymous 1992, Maud 1995). However, methyl bromide depletes the ozone layer, thus causing world-wide concern about its impact on the environment (World Meteorological Organisation 1994). Under the United Nations Montreal Protocol, methyl bromide will be banned for all uses other than pre-shipment and quarantine in the USA from 2005 (Anonymous 2001). Hence there was an urgent need to develop alternative methods of insect pest control. In response to this, The Horticulture and Food Research Institute of New Zealand Ltd (HortResearch) initiated a programme to find alternative insect control strategies for the forestry industry. Research focused on the development of heat treatments, controlled atmospheres, and the robustness and feasibility of using heat treatments commercially (Dentener et al. 1999, Dentener et al. 2001).

In this programme, huhu was chosen as an indicator species to determine the efficacy of the new treatments developed, because Cross (1991) observed that, of the insects found on export pine, the late egg and young larval stages of huhu were hard to kill with methyl bromide.

Fresh product disinfestation

Planned restrictions on the use of methyl bromide for disinfestation of fresh produce of unwanted insect pests led to increased interest in developing alternatives during the 1990s (Hallman and Armstrong 1994). Chemical alternatives such as phosphine (Williams et al. 2000) and methyl iodide have been investigated as well as non-chemical treatments based upon environmental factors such as controlled atmospheres (CA), elevated and low temperatures, and various combinations of these (Carpenter and Potter 1994, Hallman and Armstrong 1994, Denlinger and Yocum 1998). Successful quarantine treatments have been developed for disinfestation of tropical and subtropical fruits of fruit flies, based upon elevated temperatures between 40 and 50°C (Armstrong 1994). Finding successful disinfestation treatments for temperate fruit such as apples is more difficult. They require either a very long time to achieve kill at low temperatures (with or without CA) (Waddell et al. 1990) or, in the case of heat, are damaging to fruit at the time-temperature combinations required to kill insects (Smith and Lay-Yee 2000).

A very large amount of insect mortality data is required to develop new quarantine disinfestation treatments and must incorporate various treatment (or multiple treatment) time combinations, insect life-stage and fruit quality responses (Carpenter and Potter 1994). Such data is often collected on a trial and error basis, which is both time consuming and expensive. Therefore, various time-temperature thermal death models have been proposed in an attempt to provide a more systematic framework for these studies (Tang et al. 2000, Hansen et al. 2004). However, understanding how insects respond physiologically and biochemically to such extreme stresses may also enable disinfestation treatments to be developed faster and more effectively (Neven 1998, 2000, Zhou et al. 2000, 2001, Downes et al. 2003). Combining CA and heat into a single treatment is a case in point, where reduced oxygen, elevated carbon dioxide and different time-temperature combinations provide much better insect control than heat alone. Metabolic studies on *Platynota stultana*, a tortricid pest have demonstrated that elevated carbon dioxide treatments were more efficacious than reduced oxygen (Zhou et al. 2000,

2001). Yet finding the most effective combination is a major undertaking that can be assisted by a greater understanding of the insects' physiological responses (Neven 1998, 2000, Zhou et al. 2000, 2001, Neven 2003).

Insect response to stress

Typically, insects increase their metabolic rate and hence developmental rate in response to increased temperature (Gillooly et al. 2001, Gillooly et al. 2002). Within an insect's natural ecological temperature range, a useful concept is that of $Q_{10} = 2$, whereby there is often a doubling of the metabolic rate with an increase of 10°C (Schmidt-Neilson 1979). However, short to moderate temperature exposures greater than 40°C result in either rapid death, thermal wounding or no apparent effect depending on the time-temperature exposure (Denlinger and Yocum 1998). Interestingly, when insects are exposed to sublethal temperatures, heat shock proteins are induced, which confer thermo-tolerance to extreme temperatures (Parsell and Lindquist 1994).

Precisely how insects die after exposure to high temperatures is unknown. Heat injury can cause many abnormalities at the cellular level, yet cell death itself does not directly result in the death of the animal (Denlinger and Yocum 1998). Typically, higher levels of organisation, such as critical tissues and the whole organism, are most susceptible to injury that results in mortality. Thermal death models proposed by Bowler (1987) and Roti Roti (1982) both implicated the plasma membranes as the primary site. The former suggested protein damage leads to ionic leakage causing secondary and tertiary lesions and loss of cellular metabolism. In contrast, the latter focused on protein denaturation that ultimately resulted in DNA damage.

Insects typically have a much greater tolerance to hypoxic (low oxygen), anoxic (no oxygen) and hypercarbic (elevated carbon dioxide) conditions than vertebrates (Hochachka 1986, Wegener 1993). Many insects inhabit environments where they are exposed to oxygen levels of less than 1% yet they are able to function normally (Hoback and Stanley 2001). For example, the larvae of *Orthosoma brunneum* (Cerambycidae) inhabit wet decaying beech logs and display normal movement and feeding behaviour when exposed to atmospheres of 1% oxygen and up to 43% carbon dioxide (Paim and Beckel 1964). Insects employ a variety of strategies to survive hypoxia, the most common being to drastically reduce their metabolic rate but also to use alternative metabolic pathways (Hochachka 1986, Wegener 1993).

Insects can also utilise anaerobic pathways under hypoxic/anoxic conditions that result in the production of lactate (Zachariassen and Pasche 1976, Friedlander and Navarro 1979, Wegener 1993, Kolsch 2001). However, in contrast to vertebrates, anaerobic glucose metabolism does not follow the standard pathway from glucose to lactate, and results in metabolic end products in addition to lactate (Meyer 1980). During recovery from hypoxia there are often elevated levels of metabolism as an insect repays an oxygen debt (Wegener and Moratzky 1995).

Insect respiration and respirometry

Respiration measurements in animals have a long history, with the first respiration trials attributed to Lavoisier who, in 1784, floated a bell jar containing a guinea pig on mercury (Kleiber 1961). Since this time, manometric methods based on constant pressure or volume have become more sophisticated and been applied to a wide range of animals, including insects (Umbreit et al. 1964, Slama 1984). Recent advances in gas analyser technology, such as reduced internal volumes, increased sensitivity and better signal processing have greatly improved their performance. These developments have enabled a wider range of more accurate measurements of insect respiration to be made, including flow-through respirometry to elucidate different phases of gas exchange (Lighton 1991a).

Using oxygen consumption as a measure of metabolism of different sized animals enabled Kleiber (1961) to propose the mass scaling factor of 0.75. That is, $P = aM^{0.75}$, where P = basal metabolic rate (BMR), a = mass coefficient and M = mass, which has led to considerable discussion and debate (Heusner 1982, Feldman and McMahon 1983, Schmidt-Neilson 1984). The BMR (see Chapter 3) is difficult to measure as its criteria are not appropriate for many animals, thereby making inter-specific comparisons difficult (Heusner 1971, Speakman et al. 1993). Consequently, many biologists measure the standard metabolic rate (see Chapter 3), which is more pragmatic and allows better comparisons between species (Heusner 1971, Lighton 1991a, Lighton and Fielden 1995).

Insect metabolic studies have been used to investigate many different aspects of the relationship between an insect and its environment, including geographic latitude (Nielsen et al. 1999), temperature-based distribution and flight capability (Vogt and Appel 1999, Vogt et al. 2000), temperature and water availability (Davis et al. 2000), pest biology (Appel et al. 1997), and responses to anaerobic conditions (Kolsch 2001, Kolsch et al. 2002). Furthermore, respirometry and calorimetry have

provided a greater understanding of insect respiratory mechanisms, including; ventilatory patterns and their evolution (Lighton 1996, 1998, Chappell and Rogowitz 2000), spiracular gas flow and regulation (Slama 1988), energy cost and locomotion (Lighton and Feener 1989) and response to hypoxia (Wegener and Moratzky 1995).

Objectives

Huhu were chosen for these physiological studies because mortality research had demonstrated they were susceptible to heat injury, very tolerant of anoxia, but easily killed by a combination of both stresses (Dentener et al. 1999). Furthermore, there is a scarcity of information on cerambycid metabolic responses to stress, especially for larval life-stages. These physiological studies on huhu sought to determine SMR at biologically relevant temperatures and how larvae respond to stresses caused by elevated temperature and low oxygen atmospheres. The purpose was to identify changes in huhu responses that could ultimately be used for the development of commodity insect disinfestation strategies.

Objective Chapter 2:

1. To identify a suitable artificial diet and rearing conditions for huhu larvae.

The suitability of an artificial diet for huhu larvae was investigated to enable the production of uniform insects for physiological studies. This study is described in Chapter 2 and has already been published, (see Rogers, D. J., S. E. Lewthwaite, and P. R. Dentener. 2002. Rearing huhu beetle larvae, *Prionoplus reticularis* (Coleoptera: Cerambycidae) on artificial diet. *New Zealand Journal of Zoology* 29: 303-310). Consequently, the style is a little different and it contains some repetition pertaining to the biology of huhu.

Objectives, Chapter 3:

2. To determine SMR in huhu larvae and how it is affected by mass and temperature, using manometric methods.
3. To measure seasonal temperatures, which huhu are likely to be exposed to within logs.

SMR was measured in huhu larvae and pupae using manometric techniques and the effect of size and temperature on larval metabolism determined. Originally, a greater number of manometric experiments were planned at elevated temperatures with different larval sizes and durations. However, the successful development of an automated respirometry system based upon gas analysers redirected the focus of the study. Investigating the effect of elevated temperatures in the laboratory on larval metabolism poses the question; what temperatures are huhu naturally exposed to? Therefore, to provide contextual data about their natural thermal environment, seasonal temperature measurements within logs were recorded.

Objective Chapter 4:

4. To determine SMR in huhu larvae exposed to 20-40°C, and how it is affected by mass and temperature, using automated respirometry.

An automated respirometry system was constructed and calibrated. The SMR was calculated for this temperature range, mean Q_{10} , respiratory quotient (RQ, the ratio of oxygen consumed to carbon dioxide produced) and activity were measured on huhu. By necessity there was a greater focus on calibration of the components and the system as a whole, because the automated respirometry system was custom-built.

Objectives Chapters 5:

5. To determine the effect of time-temperature exposure to extreme temperature (up to 45°C) on larval gas exchange.
6. To determine if exposure to hypoxic atmospheres causes larvae to reduce their rate of gas exchange, consistent with a strategy of metabolic depression.
7. To determine if huhu larvae, while recovering from exposure to anoxia, display elevated metabolic activity, suggesting they have incurred an oxygen debt.

The automated respirometry system was used to measure gas exchange of small and large huhu larvae exposed to extreme constant temperature for extended periods. Larvae were also exposed to a temperature ramp to determine if they responded differently to a gradual increase in temperature, compared with a thermal “shock”

associated with being transferred from ambient conditions to an extreme temperature. Respiratory responses of huhu were measured during exposure to 1.8% oxygen and during recovery from a pure nitrogen atmosphere.

Objective, Chapter 6:

8. To determine if larvae use anaerobic respiration when exposed to stress caused by heat and low oxygen atmospheres.

The effects of high temperature stress on larval haemolymph pH and lactate concentrations were measured. Similarly, the effect of stresses caused by exposure to anoxic and hypoxic atmosphere treatments at different temperatures were assessed using the same haemolymph parameters.

The response of huhu larvae to stress is summarised and discussed in Chapter 7 (General Discussion). Furthermore, the relevance of this study to disinfestation research, and opportunities for future research are explored.

**REARING HUHU BEETLE LARVAE, *PRIONOPLUS RETICULARIS*
(COLEOPTERA: CERAMBYCIDAE), ON ARTIFICIAL DIET**

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Abstract

Huhu beetle larvae, *Prionoplus reticularis*, were successfully reared in the laboratory at 20°C L:D 0:24 h on an artificial diet containing pine sawdust. The larval period was reduced to about 250 days compared with at least two years in the field. Mean larval weight increased from 1.38 mg (neonate) to 3639 mg (mature larvae) after 230 days on artificial diet. Most larvae entered diapause, which was successfully terminated by ambient field conditions in season but not by laboratory chilling regimes. The diet used may be suitable for other cerambycids, and be especially useful for rearing field-collected larvae to provide adult beetles for taxonomic studies.

Keywords: Huhu, *Prionoplus reticularis*, rearing, artificial diet, development, diapause, cerambycid

Introduction

Huhu, *Prionoplus reticularis* White (Coleoptera: Cerambycidae), are common in dead wood of New Zealand softwood trees including *Pinus radiata* D. Don (Edwards 1961b). Huhu do not attack living trees although they can infest the dead parts of living trees. This type of damage often occurs in *Cupressus* and *Thuja* but is uncommon in *P. radiata*. However because eggs can be laid on freshly felled timber or on cut logs awaiting export, they are considered an economic pest of quarantine importance. Larvae can be found in older export timber. Logs are currently fumigated with methyl bromide before export to the United States market, to control huhu and other insect pests (Anonymous 1992). A substantial volume of New Zealand logs and timber products exported to a variety of North Asian markets are also fumigated with methyl bromide upon arrival to control insect pests (Maud 1995). However, methyl bromide depletes the ozone layer, causing world-wide concern about its impact on the environment (World Meteorological Organisation 1994). Hence there is an urgent need to develop alternative methods of insect pest control. In response to this, HortResearch initiated a programme to find alternative insect control strategies for the forestry industry.

In this programme huhu was chosen as an indicator species to determine the efficacy of the new treatments developed, because Cross (1991) found that of the insects found on export pine the late egg and young larval stages of huhu were hard to kill with methyl bromide. Most of the huhu life stages required for this research could be collected directly from the field. However, where very small larvae or larvae of a known age were required for physiological and dose-mortality studies it was necessary to rear them on an artificial diet in the laboratory. In this paper we describe a suitable artificial diet and laboratory rearing method for huhu larvae.

In the field, most researchers estimate the life cycle on *Pinus radiata* to be between three and five years (Morgan 1960, Edwards 1961a, Hosking 1978). Females oviposit about 250 to 350 eggs on fallen or cut logs, stumps, green sawn timber and the dead parts of live trees, and these hatch after 16 to 25 days. The larval period extends over at least two to three years, and includes an over-wintering, non-feeding final instar. After a 25 day pupation period, adult beetles exit the logs to mate. In the laboratory, larvae were reared at 20 and 25°C to determine whether elevated temperature would shorten the long life cycle observed in the field. Gardiner (1970) reared 49 species of cerambycid on artificial diets and found that most species

completed their life cycle in less than half the normal time under laboratory conditions. Further, day length has been shown to affect the development of some cerambycid larvae and other New Zealand insects (Morris 1989, Shintani and Ishikawa 1997). Hence, although the majority of the larvae were reared in complete darkness (L:D 0:24 h) to match natural conditions within timber, one sample of larvae was reared under a 16:8 h L:D photoperiod. Mature larvae (reared in complete darkness) that stopped feeding and entered diapause were exposed to a variety of environmental regimes to identify the conditions that could be used to terminate diapause.

Materials and methods**Artificial diet**

This diet used some of the same ingredients found in cerambycid diets developed by Gardiner (1970). Diet ingredients are given in Table 2.1. All diet ingredients except the ascorbic acid, mould inhibitor and vitamin solution were autoclaved at 121°C for 40 minutes (2-5 kg) or 60 minutes (6-12 kg). Following this, the hot diet mix and mould inhibitor solution were transferred to a Hobart® mixer, and blended until the temperature was between 60 and 70°C before the vitamins were added. After mixing, the diet was poured or dispensed into appropriate containers while still hot.

Table 2.1 Ingredients to formulate 2.5 kg of huhu larval rearing diet.

Ingredient	%	Amount
Wheat germ	2.43	60.8 g
Wesson's salt mix	0.81	20.3 g
Agar (food grade)	3.25	81.3 g
Cellulose powder	7.30	182.5 g
Sawdust (<i>Pinus radiata</i>)	4.87	121.8 g
Glucose	1.22	30.5 g
Yeast (roller dried)	2.43	60.8 g
Casein (lactic)	2.43	60.8 g
Sucrose	2.03	50.7 g
Water	69.79	1745.0 ml
Ascorbic Acid	0.35	8.8 g
Mould inhibitor (stock solution) ¹	1.46	36.5 ml
Vitamin solution ²	1.62	40.5 ml

¹ Composition of stock mould inhibitor solution:

methyl p-hydroxybenzoate	150 g
Sorbic acid	200 g
Ethanol (95%)	1700 ml

² (Ashby et al. 1985)

Development studies

Year one: Effect of temperature and photoperiod on development of larvae

Eggs from a single female huhu beetle collected in Tokoroa forest (New Zealand) during January 1996 provided larvae for these rearing studies. Neonate huhu larvae (10-20) were transferred with a fine sable hair brush (Haydyn 100 series, size 00) to non-ventilated plastic disposable Petri dishes (90 x 15 mm, Labserv© LB560005) containing 15g of diet. To facilitate establishment, several small notches (5 mm long and 2 mm at the widest point) were cut in the diet. Larvae were divided into 2 groups: Experiment 1 reared at either 20 (n = 69) or 25 (n = 80) \pm 1°C L:D 0:24 h or Experiment 2 at 20 \pm 1°C L:D 16:8 h (n = 43) and L:D 0:24 h (n = 30) to determine optimal rearing conditions. When larvae weighed about 8 mg (18 d for Experiment 1) and 17 mg (31 d for Experiment 2) they were transferred (one per container) to ventilated 75 ml specimen vials (Labserv© 30002) containing 35 g of diet. Larvae were weighed at 14-day intervals after being transferred to individual containers until pupation. Data for insects that died during the weighing period were included up to the last date that they were weighed alive. Once larval weight reached 2000-2500 mg, they were transferred to ventilated 400 ml PTFE square containers (Cospac NZ Ltd.) with 200 g of solid diet.

Year two: 20°C rearing

Huhu egg batches were collected from under the bark of recently felled *Pinus radiata* logs in Riverhead forest (Auckland, New Zealand) during February 1997. A sample of 30 neonate huhu (< 24 h), from three different egg batches were weighed to provide an indication of the mean initial weight.

Huhu larvae were mass-reared as described above, 10-20 per Petri dish. One hundred of these were extracted from diet when 13 days old, weighed and individually inoculated onto diet in non-ventilated Petri dishes (90 x 15 mm) as described above. The larvae originated from 5 different egg batches, and progeny from each batch were treated as replicates (n = 16 - 24 larvae per replicate). Larvae were removed weekly from the diet for weighing, and subsequently returned to their diet or transferred to fresh diet in a new Petri dish as required. After 111 days the larvae reached an average weight of 852 \pm 39 mg, (SEM) and required larger rearing containers. They were placed individually in deeper ventilated Petri dishes (90 x 25 mm Labserv© LB560005). The deep Petri dishes were used in preference

to the larger volume containers used in year one, because the larvae were unable to utilise all of the diet within a large container, and also because it was easier to observe moulting in the Petri dish. During weighing, both larva and Petri dish were checked for any sign of larval moulting. All moulted head capsules and skins observed were recorded and removed.

Diapause

During preliminary studies we observed mature huhu larvae (approx. 4000 mg) that had stopped feeding, entered diapause but failed to pupate. To identify factors that could terminate diapause, groups of diapausing huhu larvae ($n = 26$ per group) that had been reared at 20°C L:D 0:24 h were exposed to different environmental conditions, including field conditions, CO₂ and various chilling regimes. In all cases larvae were contained individually within ventilated 90 x 25 mm Petri dishes with artificial diet.

Control larvae ($n = 26$) were maintained at 20°C L:D 0:24 h for comparison. The first group of larvae ($n = 26$) were exposed to field conditions by being placed in a 330 x 220 mm Styrofoam container ("6-pack chilly bin") under a shady tree on a North facing slope at the Mt. Albert Research Centre, Auckland on April 25 1997. The temperature was recorded hourly within the container with an Orion Tinytalk® 11 data logger until diapause was broken. The second group of larvae ($n = 26$) were exposed to 100% CO₂ for 10 days at 20°C L:D 16:8 h and then returned to the standard rearing conditions with artificial diet. The final three groups of larvae (26 per treatment) were exposed to three different artificial temperature regimes that could potentially break diapause: 1) 8 weeks at 10°C; 2) 1 week at 10°C followed by 8 weeks at 4°C then 1 week at 10°C; 3) the temperature was decreased in 2°C increments per week (i.e. 1 week at each of 18, 16, 14, 12, 10°C) to 10°C, followed by 8 weeks at 8°C then increased to 20°C using 2°C increments per week. During all temperature regimes the pre- and post-treatment temperature was 20°C and photoperiod L:D 0:24 h.

Adults

Adult beetles that developed from larvae reared on artificial diet were kept at 15 ± 1°C until they could be paired. Each pair of adults was placed in a perspex container (500 x 356 x 200 mm, Ashby et al. 1985) and held at 20 ± 1°C L:D 16:8 h. Refuges of curved pine bark and freshly cut cross-sections of pine logs (typical of observed

oviposition sites, approx. 150 mm diam. and 100 mm thick) were supplied for oviposition.

Statistics

Mean weights and 95% confidence intervals for year one were calculated for each weighing date using Microsoft Excel™ version 5a. Development curves were fitted and plotted with Microcal™ Origin™ version 4.1 using the sigmoidal fit function that uses the Boltzman equation. Year two mean weights were plotted using the Loess smoothing procedure available in S-Plus (Statistical Sciences Inc. 1991). Hourly datalogger recordings made during larval exposure to field conditions were used to calculate weekly mean, maximum, and minimum temperatures.

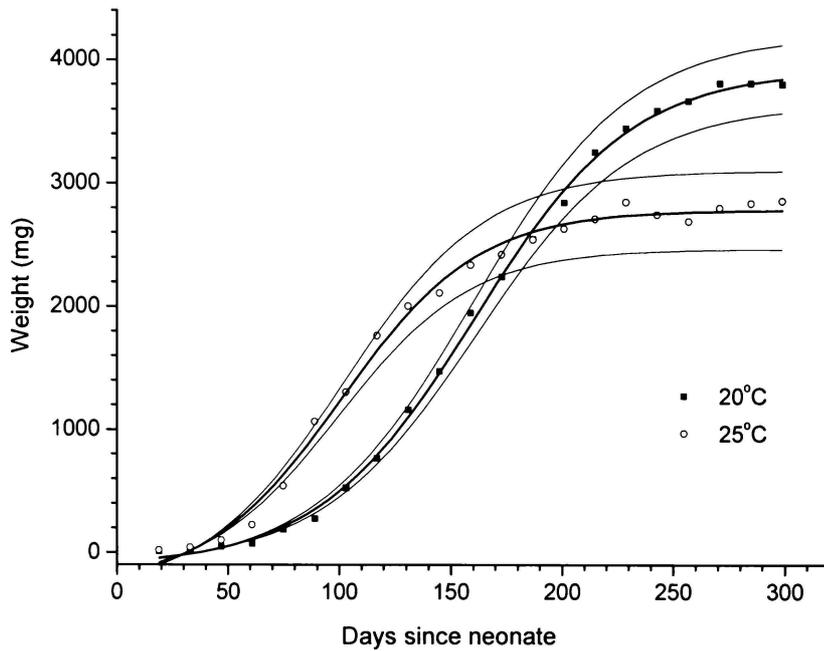
Results and Discussion

Artificial diet

Preliminary artificial diet studies (Rogers et al., unpublished data) indicated that huhu larvae could feed and gain weight on agar - wheat germ - casein based diets with the addition of ground pine sawdust. These diets were based on other cerambycid diets developed by Gardiner (1970); Payne et al. (1975); Viedma et al. (1985). Gardiner (1970) found that the addition of pulverised plant material increased the acceptance of the diet by young cerambycid larvae. The final artificial diet developed (Table 2.1) contained 5% sawdust (*Pinus radiata*) and was modified (reduced water content) from the most successful diets tested during preliminary studies.

Eighty-nine percent of neonate larvae (n = 266) successfully established on the artificial diet. They were observed to feed and establish tunnels within 5 minutes of inoculation. Establishment was best when the diet had been poured into the dish and there was still a clear margin between the edge of the dish and the diet with small notches to facilitate entry. Huhu larvae fed and gained weight on the artificial diet. Although the larvae are not cannibalistic, observations have shown that they are strongly territorial and will bite other larvae. Therefore larger larvae were reared individually in large containers that allowed the larvae to bore into solid diet (poured when liquid) and form galleries and chambers, mimicking their natural behaviour in timber (Edwards 1961a). All larval growth curves were sigmoidal in shape (Figures. 2.1-2.3) and typical of insect development over multiple instars (Hilbert 1995).

Figure 2.1 Year one, Experiment 1, mean weight (bold lines) and 95% CI of huhu larvae reared at 20 and 25°C on artificial diet.



Edwards (1959) found huhu had a wide host range including a variety of New Zealand native and non-native tree species. This implies that huhu are naturally generalist feeders and may in part explain why huhu grow well on artificial diets. The diet may also be suitable for other cerambycid larvae and if so, be especially useful for rearing field-collected larvae to provide beetles for taxonomic identification. It has been successfully used to rear small numbers of larval *Arhopalus tristis* (F.) (burnt pine longhorn) and *Oemona hirta* (F.) (lemon tree borer) to adulthood, (Max Suckling and Qiao Wang, pers. comm. 1998, respectively).

Laboratory reared adults were paired and placed in oviposition cages. However, no eggs were collected because only small numbers pupated spread over several months and aggressive males often killed females.

Figure 2.2 Year one, Experiment 2, mean weight (bold lines) and 95% CI of huhu larvae reared at 20°C, L:D 16:8 h = “light” and L:D 0:24 h = “dark” on artificial diet.

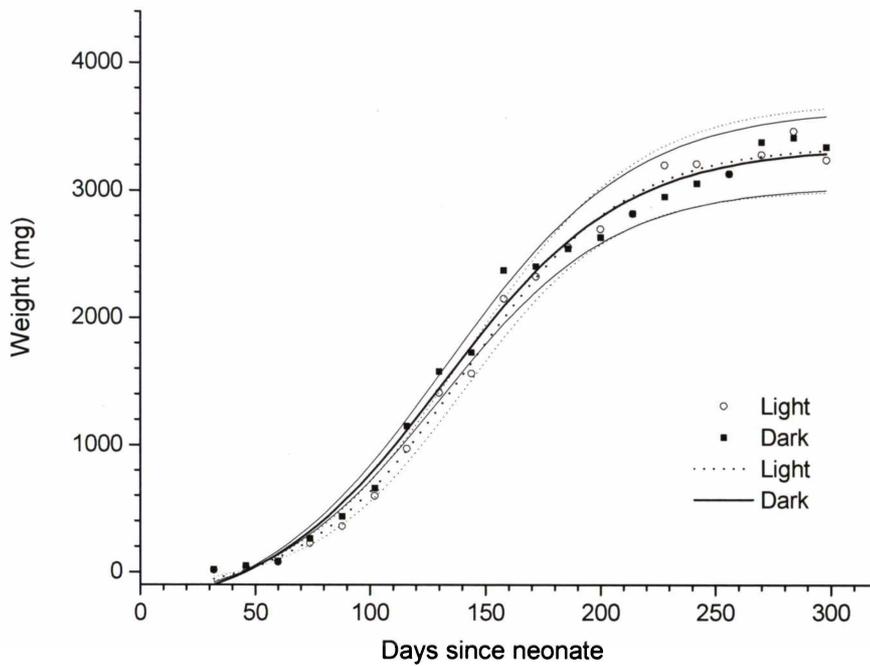
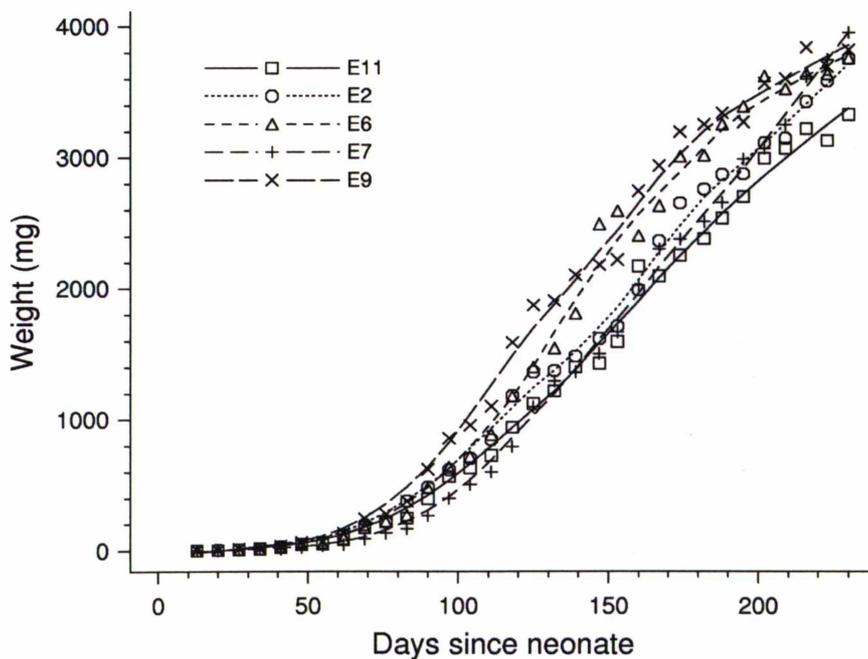


Figure 2.3 Year two, mean weight of huhu larvae from 5 different egg batches reared at 20°C on artificial diet. E2, E6, E7, E9, E11 denote different egg batches.



Year one: Effect of temperature and photoperiod on development of larvae

Huhu larvae reared at 25°C initially grew faster than larvae reared at 20°C (Figure 2.1: Experiment 1), indicating that maintaining larvae at elevated rearing temperatures could significantly reduce the generation time. However after 185 days the mean weight of the huhu reared at 20°C was greater than the huhu reared at 25°C and continued to increase, whereas the rate of weight gain of larvae at 25°C levelled off (Figure 2.1). After 418 days larval mortality was 42% at 25°C compared to 5.8% at 20°C; the larvae were noticeably smaller and none had pupated (Table 2.2). Elevated rearing temperatures have been shown to have a deleterious effect on other New Zealand insects such as the native leafroller *Ctenopseustis obliquana* (Walker) that will not lay fertile eggs at 22 and 25°C (Clare and Singh 1990). In contrast Iglesias (1989) reared a variety of Spanish cerambycids including *A. tristis* on artificial diet and found they grew faster and with reduced mortality at 25°C compared with 20°C, which may reflect climatic or species differences.

There was no apparent difference in growth between huhu reared at 20°C L:D 0:24 h, and those exposed to a photoperiod of L:D 16:8 h (Figure 2.2: experiment 2). After 417 days, more of the huhu exposed to L:D 16:8 h had pupated and mortality was higher (Table 2.2) but further work is required to confirm these observations. Since huhu larvae are adapted to dark conditions inside timber, further rearing was carried out in complete darkness.

Year two: 20°C rearing

Growth data for the 100 huhu larvae reared at 20°C are presented in Figure 2.3, and a development summary in Table 2.2. Mean larval weight (of the 5 replicates) increased from 1.38 mg (SEM: 0.019, neonate, n = 30) to 3639 mg (n = 79, SEM: 130) after 230 days at 20°C (Figure 2.3), which was when the first prepupae were recorded. Larval weight continued to increase after 230 days, but the calculations of means were skewed by the larvae that ceased to grow and entered the prepupal or diapausing phase. Figure 2.3 shows distinct differences in weight gain between larvae from different egg batches. However, further studies are required to statistically quantify these differences. Year two larvae reared at 20°C grew faster than year one larvae. While there could be genetic reasons for this it may also be due to improvements in the rearing methods. During the first year we used large 400 ml rearing containers in an attempt to mimic the natural environment, allowing

the larvae to bore into a large volume of diet. Year two larvae were reared entirely in disposable Petri dishes and had access to a much smaller amount of diet that was replenished more often. The latter may have provided better nutrition, because fresh diet was always available. Year two larvae had a higher level of mortality than year one larvae, but more frequent handling of smaller larvae contributed to this. Our studies suggest that the length of the huhu life cycle at 20°C in the laboratory could be reduced to approximately 330 days.

Pupation and diapause

While a small number of larvae pupated, the majority entered diapause, during which mature larvae cease feeding and their weight remains static, or even decreases. It was difficult to distinguish larvae in diapause from those still feeding but growing slowly, and consequently to determine the onset of diapause with any precision. Larvae held at 20°C in complete darkness remained in diapause for a year with only the occasional larva pupating. Gardiner (1970) also found that a high percentage of laboratory reared cerambycids entered diapause. While a proportion of *Graphisurus fasciatus* (De Geer) pupated, the majority remained in diapause in the final instar. When exposed to a cold treatment of 8 weeks at just above 0°C, development was completed when the larvae were returned to ambient laboratory temperatures. In the field Edwards (1961a) observed that last instars that failed to pupate by mid summer ceased feeding and passed the cold winter months in diapause. These dormant larvae resumed activity about mid August, when they built a pupal chamber, entered the prepupal phase without feeding and then pupated. Edwards (1961b) believed that it was unlikely that photoperiod was responsible for termination of diapause, and suggested that it was most likely to be due to the annual temperature rise in August.

We observed some mature field-collected larvae were induced to pupate following a CO₂ disinfection treatment (Dentener et al. 1999). Paim and Beckel (1962) found that O₂ levels could drop below 2% and CO₂ increase to 15% within rotting beech logs inhabited by cerambycid larvae. We postulated that increased levels of CO₂ may be present within huhu larval chambers in *Pinus radiata*, and this could influence diapause. However, exposing laboratory reared diapausing huhu larvae to artificial CO₂ treatment or different artificial chilling regimes did not terminate diapause in our experiments. In contrast, laboratory reared diapausing larvae exposed to field conditions from 25 April 1997 all pupated during October and

November 1997, following the same timing Edwards (1961b) had observed with wild huhu in the field. Figure 2.4 shows the temperature range experienced by the larvae during this period. Mean temperatures for July and August 1997 averaged approximately 10°C, which was similar to the artificial regimes we used to try to terminate diapause. Perhaps larvae require longer periods of decreasing temperatures to terminate diapause. Alternatively, photoperiod may be an important factor, since the Styrofoam container we used in the field was translucent. Shintani and Ishikawa (1997) reported that either chilling at 10°C for 30 days or daylength longer than 13.5 hours terminated diapause in the yellow-spotted longicorn beetle (*Psacothaea hilaris* (Pascoe)).

Table 2.2 The effect of environmental conditions on huhu development.

	Conditions Temp./L:D	n ¹	Age ² (days)	% Mortality	% Pupated	Larval weight ³ ± SEM (mg)
Year 1						
Experiment 1	20°C L:D 0:24 h	69	418	5.8	14.5	4768 ± 189
	25°C L:D 0:24 h	80	418	42.0	0.0	3534 ± 230
Experiment 2	20°C L:D 0:24 h	30	417	10.0	6.7	4222 ± 278
	20°C L:D 16:8 h	43	417	18.6	27.9	4233 ± 268
Year 2	20°C L:D 0:24 h	100 ⁵	286	23.0	23.0 ⁴	4295 ± 130

¹ the number of small larvae (<10mm) transferred to fresh diet

² age from neonate

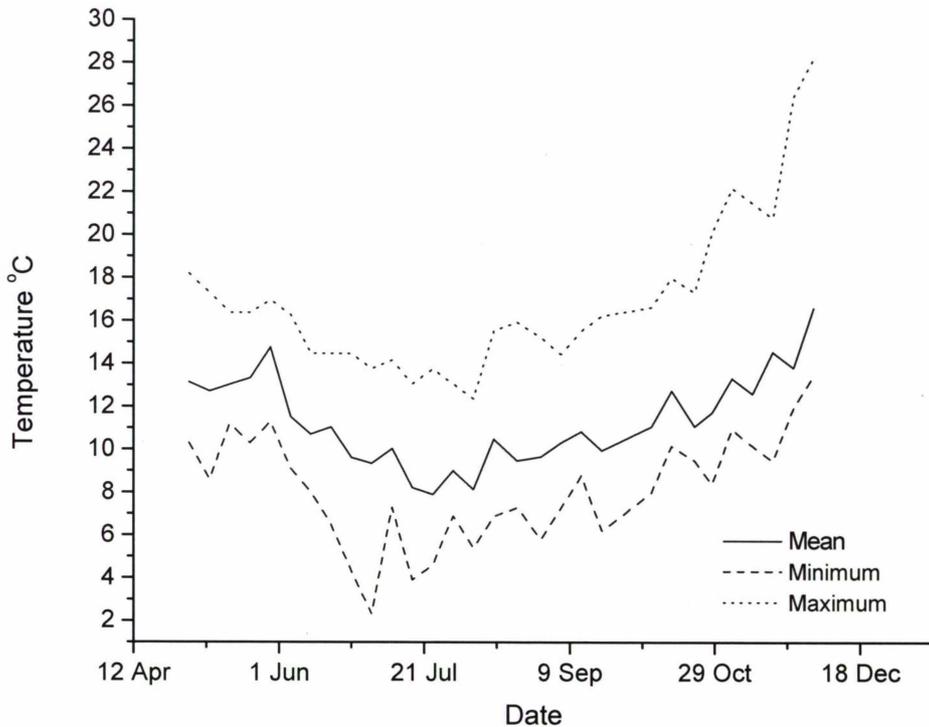
³ mean larval weight of the remaining larvae that had not pupated

⁴ includes prepupae

⁵ combined from 5 replicate egg batches

After 286 days a total of 23% of the year two larvae had become prepupae. However, all but one prepupa originated from two of the five egg batches. The combined percentage of larvae that became prepupae from these two egg batches was 50% (n = 44, E11 and E9) compared with 1.8% (n = 56, E2, E6 and E7) from the other three egg batches. Since the egg batches were probably from five different females, this suggests that diapause may also be influenced genetically or by some environmental effect on the female.

Figure 2.4 Weekly mean, minimum and maximum temperatures experienced by laboratory reared huhu exposed to ambient outside conditions.



Conclusion

The artificial diet developed in this research is suitable for rearing huhu larvae for physiological and dose-mortality studies. Larvae should be reared at 20°C in preference to 25°C, as the higher temperature ultimately results in higher mortality and reduced larval weight. The fact that many larvae entered diapause prevented a full evaluation of the diet and rearing procedures, including the production of fertile adult beetles. Further work is required to identify the factors that control diapause and to develop suitable adult cages and substrates for huhu oviposition.

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MEASURING RESPIRATION IN HUUH USING MANOMETRIC METHODS

Introduction

Oxygen consumption provides a good indication of an animal's metabolic rate, contingent on the use of appropriate measurement criteria (Schmidt-Neilson 1984, Slama 1984, Lighton 1991a). Metabolic rate is affected by mass, activity, feeding status, circadian rhythms, body and ambient temperatures, and developmental stage (Heusner 1971, Lighton 1991a). Kleiber (1961) defined criteria for the measurement of basal metabolic rates (BMR); "mature animals in the post-absorptive state and measured in the range of metabolically indifferent environmental temperature at rest, or at least without abnormal activity". Other descriptions such as standard metabolic rate (SMR) and metabolism defined in terms of resting, routine, active, and maximum have been used and the varied definitions have been widely discussed (Heusner 1971, Lighton 1991a, Speakman et al. 1993, Chaabane et al. 1999, Clarke and Fraser 2004). To make intra- and interspecific comparisons it is vital to have a reliable measurement of metabolism with adherence to defined practical criteria. SMR is commonly used by entomologists and fish biologists; the animal should be motionless (or show minimal movement), fully exothermic and at a known temperature (ideally controlled). Furthermore, SMR sensitivity to temperature should be determined with appropriate live mass (plus other relevant measures of mass) (Heusner 1971, Lighton 1991a, Lighton and Fielden 1995). Heusener (1971), in reviewing Klieber's criteria, concluded that, in defining a metabolic standard, greater emphasis needed to be based on reproducibility rather than the lowest level of metabolism.

Manometric methods have a long history as an investigative tool for measuring gas exchange in biological organisms, for review see Umbreit et al. (1964). The most widely known equipment is the Warburg apparatus, which uses the direct and indirect methods of respirometry. It can be used with different gas mixtures but is subject to changes in ambient pressure, and exact calibration of the vessel is required (Slama 1984). Manometric techniques can be miniaturised and the most sensitive is the Cartesian diver that can be used with a few cells and can detect

volume changes less than 1 nl. Divers are difficult to make and use, but have given rise to a variety of microrespirometric techniques, some of which have automated data collection (Lovtrup 1973). Respirometers based on the direct volumetric principle, such as the Gilson respirometer, were popular for measuring insect metabolism due to their reliability and convenience (Scholander and Iversen 1958, Gilson 1963, Slama 1984). This method has a respiration chamber with a carbon dioxide absorber and is connected to a compensatory chamber of the same volume via a manometer. After oxygen is consumed the system is balanced using a measuring device, which provides a direct measurement of the oxygen consumed per unit time (Umbreit et al. 1964). This method has several advantages over other manometric methods in that the volume of the vessel does not need to be known and the system is immune to small variations in atmospheric pressure and temperature.

The effects of size and temperature on animals have been the focus of many biological studies and have led to two, much debated principles of biological similarity. The 0.74 mass-scaling exponent and temperature sensitivity of $Q_{10} = 2$ for metabolism of all animals (Kleiber 1961, Heusner 1982, Feldman and McMahon 1983, Schmidt-Neilson 1984, Gillooly et al. 2001, Clarke and Fraser 2004). Mass-scaling metabolic equations for insects and related invertebrates also display some commonality but vary depending on the measurement methods used (Lighton and Fielden 1995, Addo-Bediako et al. 2002). Temperature sensitivity of SMR, as shown by Q_{10} values, is of value in determining an insect's preferred temperature range, foraging and general energetics and adaptations to extreme temperature fluctuations (Lighton and Bartholomew 1988, Lighton 1989, May 1989, Vogt and Appel 1999, Nielsen et al. 2003). Furthermore, temperature affects insect discontinuous gas exchange cycles, development and pest phenology (Lighton 1996, Denlinger and Yocum 1998, Chappell and Rogowitz 2000, Neven 2000, Gillooly et al. 2002, Neven 2003).

The primary objectives of this study were:

2. To determine SMR in huhu larvae and how it is affected by mass and temperature, using manometric methods.
3. To measure seasonal temperatures, which huhu are likely to be exposed to within logs.

Preliminary experiments were conducted to determine appropriate measurement criteria such as degree of starvation, adjustment time in the respirometer, and methane production. Originally, a greater number of manometric experiments were planned at elevated temperatures with different larval sizes and durations. However, the successful development of an automated respirometry system based upon gas analysers redirected the focus of the study. An overall goal of these studies was to determine the physiological response of huhu to extreme temperatures ($> 40^{\circ}\text{C}$) such as those used in commodity disinfestation. Therefore, to provide contextual data about the natural thermal environment huhu larvae are exposed to, seasonal temperature measurements within logs were also studied.

Materials and methods

Insects

Huhu beetle eggs, larvae and pre-pupae were collected from logs and stumps in a commercial pine plantation (Carter Holt Harvey Forests Ltd, Riverhead, Auckland, New Zealand) (Lester et al. 2000, Rogers et al. 2002). The hatched and field-collected larvae were reared at 20°C on artificial diet, L:D 16:8 h, as described by Rogers et al. (2002). Field-collected larvae were reared in the laboratory for a minimum of 3 weeks prior to physiological measurements. Unless otherwise explicitly stated, actively feeding insects were starved overnight (12-16 h) in 10 ml disposable plastic syringes (Becton Dickinson (BD), Singapore) and only used for a single experiment, which took place during daylight. Neonate larvae (< 24 h old) were allowed to hatch in the laboratory at 20°C from field-collected eggs.

Equipment

Manometric compensatory respirometers were constructed as depicted in Figure 3.1, using the direct volumetric principle (Scholander and Iversen 1958). The respirometry chamber consisted of a 10 ml disposable syringe connected to a 3-way stopcock (Discofix®, Braun, Melsungen, Germany) and a 5 ml syringe (BD, Singapore) containing a carbon dioxide absorbent. Two more stopcocks connected to the chamber connected a 2 ml micrometer syringe (2 µl graduations, Gilmont® Instruments, Barrington, USA) and the barrel of a 1 ml disposable syringe that was used as a vent. A 23-gauge hypodermic needle (BD, Singapore) connected the system to manometers constructed from polyethylene tubing (0.5 mm i.d., 0.8 mm o.d.; Critchley Electrical Products Ltd., Auburn, Australia), mounted in a “U” shape on laminated graph paper with plasticine (Bonza Plasto®, Auckland, NZ) and attached to solid PVC (180 x 35 x 5 mm). Modified Krebs' (1951) manometer fluid was used to fill the manometers, consisting of 44 g.l⁻¹ anhydrous sodium bromide, 2 g.l⁻¹ of Triton® X-100, 0.3 g.l⁻¹ of acid fuchsin and purified water. The standard Krebs' fluid containing only 0.3 g.l⁻¹ of Triton X moved poorly in the narrow bore polyethylene tubing, tending to break up and form "beads" of fluid within the tube. The compensatory side of the respirometer was connected to the manometer via a 23-gauge needle as described above and consisted of a 10 ml disposable syringe and a stopcock (Figure 3.1). An additional stopcock and a 5 ml disposable syringe were added later to allow larger volumes on the insect side of the manometer to be matched.

The respirometers were immersed in a cooled 40 l water bath, originally from a Gilson differential respirometer (Gilson 1963), modified by fitting a fuzzy logic plus PID temperature controller (model BTC-9200, Brainchild Electronic Co. Ltd., Taiwan). Furthermore, the original stirrer was replaced with a submersible aquarium pump (Rena™ C40, France, 540 litre/h), and allowed space for 11 respirometers within the water bath. The temperature uniformity within the water bath, excluding the zone within about 20 mm of the heater element, was $\pm 0.06^{\circ}\text{C}$ at 20°C , measured using a digital reference thermometer (Fluke Model 2180, Washington, USA). A 38-litre circulating water bath fitted with rubber mountings to minimise vibration (model W38-ZD, Grant Instruments, Cambridge, UK) that housed 8 respirometers was used for measurements at elevated temperatures (Figure 3.2).

Respiration measurements

The water bath was allowed to equilibrate for at least 1 h before experiments commenced. Each respirometer was then checked for leaks by closing the stopcocks to isolate the system from the external atmosphere, pressurising the respiration chamber by adjusting the micrometer syringe and observing the manometers. Each huhu larva was weighed prior to being placed in the respiration chamber. Insect weight was used to calculate larval volume enabling the compensatory and respirometry chambers to be matched. The compensatory chamber minimised the effect of small changes in temperature and atmospheric barometric pressure.

The volume of huhu larvae was calculated using the Archimedes principle, i.e. when a body is immersed in a fluid (liquid or gas) it experiences an upthrust equal in magnitude to the weight of the fluid displaced (Whelan and Hodgson 1972). Consequently, when an object is suspended from a thread and immersed in water the increase in weight recorded by a pan balance placed under the entire apparatus is equal to the volume of displaced water. Different weight huhu larvae ($n = 40$) were immersed in distilled water and any adhering air bubbles were removed with a fine paintbrush. The recorded weight was used to calculate the huhu volume based on the density of water.

Figure 3.1 Diagram of the manometric compensatory respirometry system.

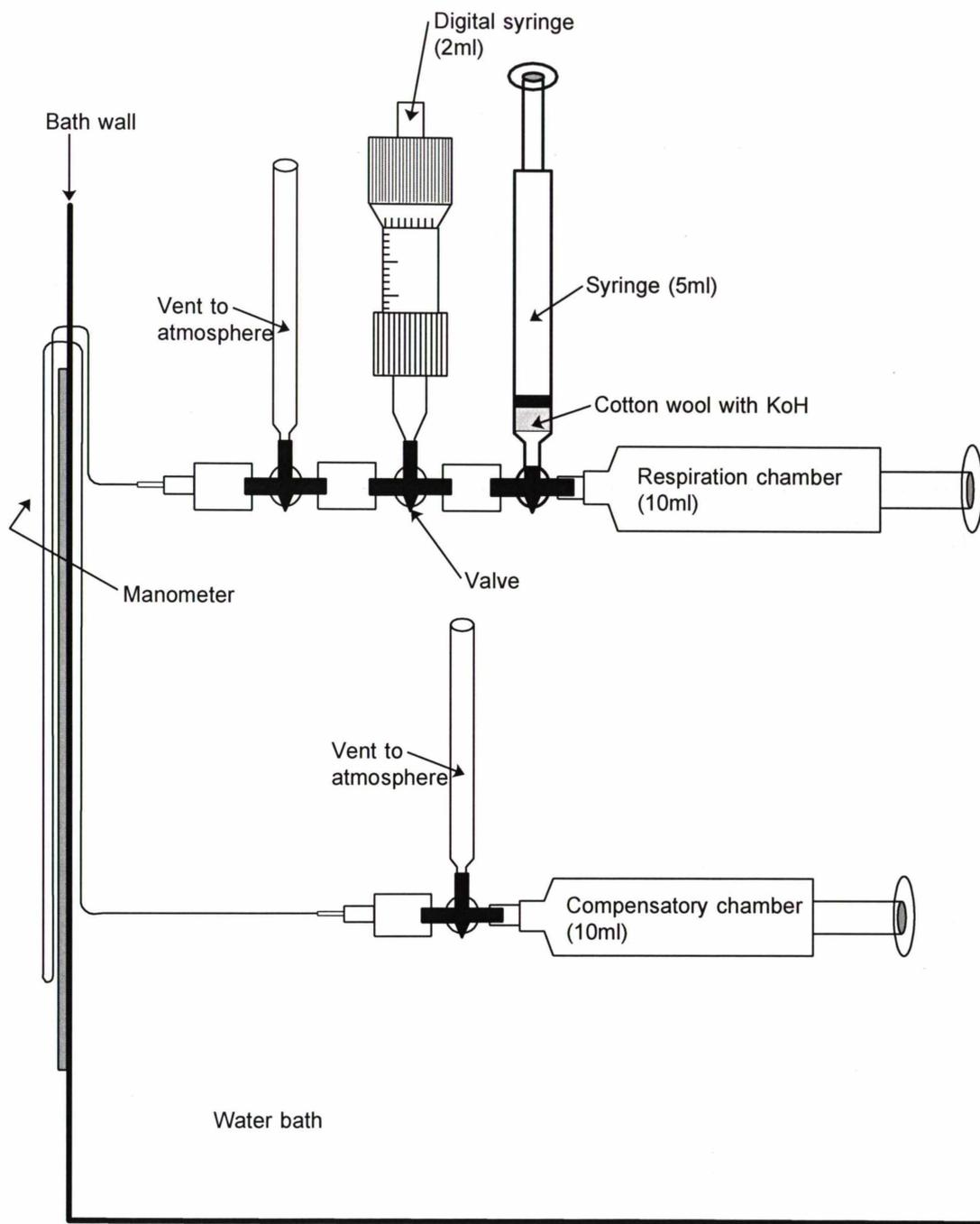


Figure 3.2 Assembled respirometers in a water bath, showing the manometers on the front and the mechanism allowing the respirometers to be independently lowered into the water.



Potassium hydroxide (180 μ l, 20% w/v) was placed on a 5 mm length of dental wick (Robinsons, Switzerland) to absorb carbon dioxide and inserted into the 5 ml syringe of each respirometer (Umbreit et al. 1964). Huhu larvae were placed into each respirometry chamber and immersed in the water bath at 30 s or 1 min intervals. The stopcocks were left open to the atmosphere for 30 min (later 60 min) to allow the huhu larvae to adjust to the temperature and their new environment. The stopcocks were then sequentially closed using the same time interval to provide enough time for subsequent measurements to be recorded from each respirometer. As oxygen was consumed within the respiration chamber the manometer fluid rose on that side of the manometer. The manometer was then adjusted after predetermined time intervals to the zero-difference position by decreasing the volume of the micrometer syringe (i.e. increasing the digital reading to maintain constant pressure). Consequently the volume change recorded by the micrometer syringe was equivalent to the volume of oxygen consumed during that time interval, assuming complete removal of carbon dioxide and that no other gases were produced. These measurements were corrected using volume changes measured by an identical empty respirometer.

Oxygen consumption measurements were corrected to standard temperature and pressure (STP, 0°C and 760 mm Hg) according to;

$$\dot{V}_{O_2} = \frac{273V_i P_M}{P_N (273 + T)}$$

where; \dot{V}_{O_2} is the STP-corrected oxygen consumption, V_i is the measured volume of oxygen, P_M is the measured barometric pressure, P_N is standard barometric pressure and T is the temperature in °C (Weast et al. 1985). Values were then converted to appropriate SI units to yield specific oxygen consumption rates (\dot{M}_{O_2} , $\mu\text{mol.kg}^{-1}.\text{s}^{-1}$, wet weight) (Harrison et al. 1991, Banks et al. 1995). Huhu \dot{M}_{O_2} rates were also expressed in nmol.s^{-1} when regressed on mass.

Developing measurement conditions

Actively feeding huhu larvae were starved for 48, 24, 12-16 h (overnight) or not starved (n = 30, 30, 10 and 10 respectively, mean weight 2.58 ± 0.06 g, \pm SEM), prior to placement in the respirometry chambers. The larvae were allowed to adjust to the chambers at 20°C for a period of 30 min before the commencement of the experiment. Oxygen consumption measurements were recorded from each larva every 5.5 min for 38.5 min, which equated to 7 measurements per insect. The chambers were then opened to the air and the syringe pumped back and forth to fill the chamber with ambient air. After 2 h the valves were closed and the measurement cycle repeated again. Two measurement cycles were completed for all larvae and an additional third cycle for two groups of 10 huhu; starved overnight and not starved. Elapsed time was recorded for each experiment starting from immersion of the chambers in the water bath.

Larvae

Actively feeding huhu larvae ranging in weight from 0.30 g to 3.27 g were starved overnight, placed in the respirometry chambers at 20°C and allowed to adjust to their new environment for 60 min. Two measurement cycles were completed for each larva as described above and a mean \dot{M}_{O_2} calculated and expressed as either a specific rate ($\mu\text{mol.kg}^{-1}.\text{s}^{-1}$) or per larva (nmol.s^{-1}). Measurements were made on 10 larvae at a time on 8 different occasions (n = 80 larvae).

Respirometry on neonate larvae less than 24 h old and weighing 1.38 ± 0.019 mg (\pm SEM, n = 30) was carried out over a 24 h period in groups of 10 larvae per chamber on three separate occasions (n = 30 groups of insects). Mean \dot{M}_{O_2} was calculated based upon readings taken during the 24 h period. Oxygen consumption of huhu larvae exposed to red fluorescent light (dark) was measured for 20 larvae at 20°C with a mean weight of 1.74 ± 0.11 g (\pm SEM) using the standard conditions

described above. The \dot{M}_{O_2} values obtained were compared with those from huhu with a similar weight, 1.82 ± 0.07 g (\pm SEM; n = 45).

Pupae

Pupation dates of field-collected pre-pupae were recorded to enable measurements to be made on known age pupae. The sex of each pupa was determined based upon the length of their antennae (Edwards 1956). Oxygen consumption was measured as described for larvae at 20°C for 10 male and 10 female pupae on two occasions 10 days apart. Observations of the typical rhythmic jerking movements made by huhu pupae were recorded when noticed, however, the measurement regime, coupled with the number of huhu, made observation of all movement difficult.

Elevated temperatures

Oxygen consumption was measured at 20, 25, 30, 35, 40, 42 and 45°C (n = 30, 18, 24, 30, 18, 6 and 17 respectively) as described above. The mean weight of the huhu larvae measured at 20-42°C was 0.58 ± 0.02 g (\pm SEM). At 45°C, two groups of larvae were treated; mean weights 0.277 ± 0.016 g, and 1.85 ± 0.13 g (\pm SEM). The smaller size of the larvae measured at elevated temperatures necessitated a longer duration between measurements. Therefore, measurements were made every 15 min for 1 h, i.e. elapsed times of 75, 90, 105 and 120 min. At 45°C, measurements were extended to cover 210 min. Water bath temperatures were verified either directly with a traceable reference thermometer (RT-200, Industrial Research Ltd, Wellington, NZ) or with a 0.1°C graduated mercury glass thermometer calibrated to the reference thermometer.

Activity was assessed using a time-based, instantaneous sampling method (Martin and Bateson 1986). At 15 min intervals, larval movement was categorised as either “immobile” (no movement), “some” movement (often small lateral head movement and intermittent) or “moving” (continuous rhythmic movement). At temperatures where insects could remain active ($\leq 35^\circ\text{C}$), movement was usually consistent for the entire experiment, therefore individual huhu were assigned to movement categories.

Methane production

Ten huhu larvae (mean weight 2.56 ± 0.08 g, \pm SEM) were confined to sealed 10 ml disposable syringes (BD, Singapore) for 1 h at 20°C. Samples (5 μ l) of gas were withdrawn from the syringes and injected into a capillary gas chromatograph

(Hewlett Packard 5890, with a J&W DB-1 column, 30 m x 0.25 mm i.d. with a 0.25µm coating) fitted with a FID detector. The resulting peaks were integrated and amounts of methane in the sample gas calculated from the relationship of known methane standards to peak area. The oxygen in the sample caused a small peak at the same position as methane because neither gas were retained by the column, therefore sample methane volumes were corrected for the estimated amount of oxygen in the sample.

Temperatures within logs

The temperature within *Pinus radiata* logs where huhu larvae are typically found was recorded in three logs using Tinytalk II data loggers connected to 150 x 6 mm PT100 probes (Gemini Data Loggers, UK). The probes were inserted 120 mm into the butt end of logs, 100 mm from the edge and the loggers maintained from 29 April 1998 to 31 August 2000 in Riverhead forest (Auckland NZ). All logs were in contact with the ground on a south facing slope with minimal vegetation and received some direct sunlight during part of the day. The loggers were set to record the temperature hourly, from which daily means, minima and maxima were calculated for each logger. Finally, combined weekly means, minima and maxima were determined. An indication of the buffering effect on temperature provided by a log was measured in Hawke's Bay during January 2004. A temperature probe was inserted into a damp *P. radiata* log (320 mm diam. x 1100 mm) as described above and positioned in the shade of some mature oak trees. The recorded temperatures were compared to air temperature recorded by the HortResearch meteorological station approximately 100 m away.

Statistics

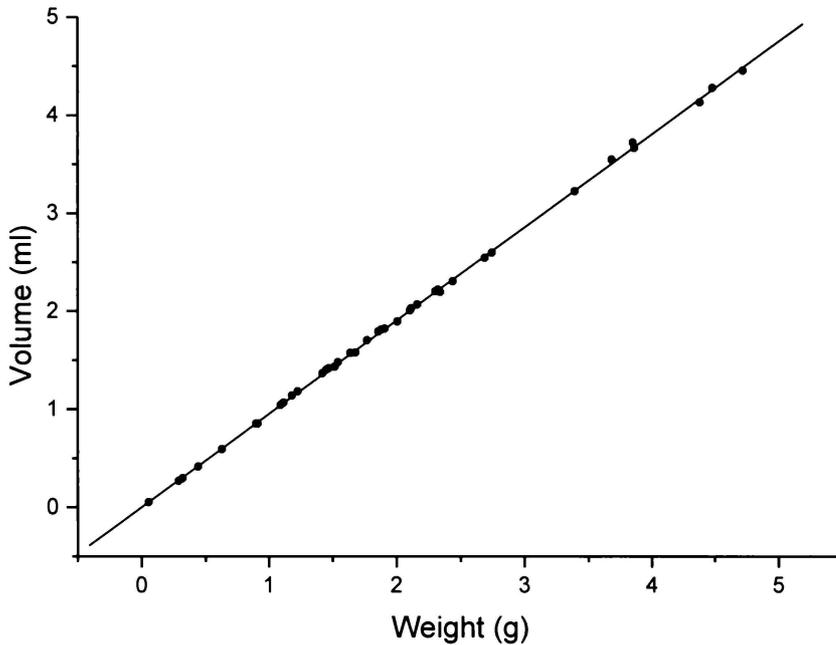
Data were linearized when necessary using a Log₁₀ transformation. Means, standard errors and standard deviations were calculated in either Excel or Minitab® (Microsoft® Excel 2000; Release 14, Minitab Inc., USA). One-way ANOVA and exploratory data analysis were performed in Minitab®. Regression lines were fitted and plotted using Origin™ (Version 5.0, Microcal Software Inc., MA, USA).

Results

Developing measurement conditions

Huhu larvae were slightly denser than water, with volume displaying a strong linear fit to increasing live weight (Figure 3.3).

Figure 3.3 The relationship between the weight and volume of huhu larvae. The regression equation is $\text{Volume} = 0.955\text{Weight} + 0.00024$, $P < 0.0001$, $r^2 = 0.999$.

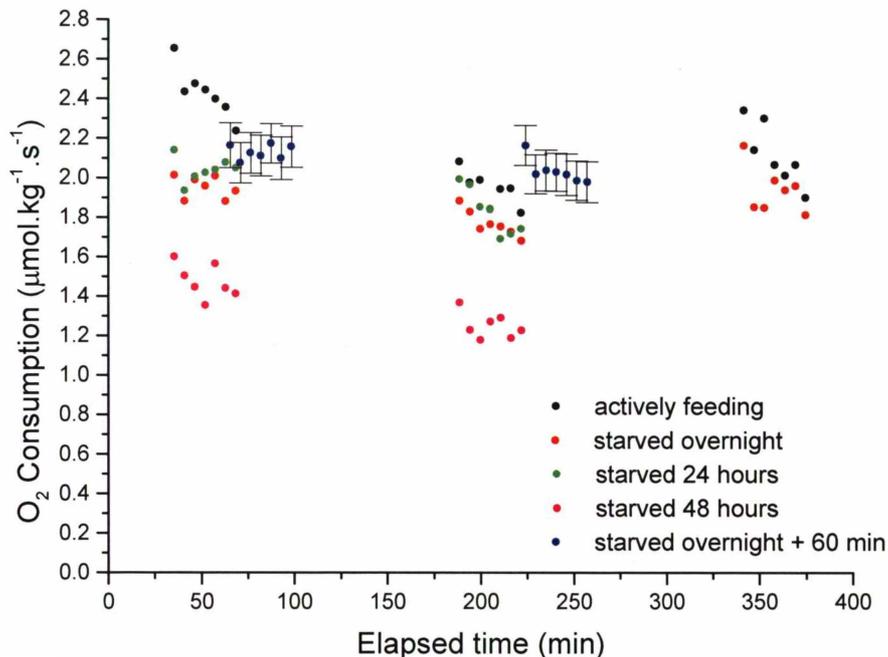


When actively feeding huhu larvae were placed directly in the respiration chambers there were noticeable decreases in oxygen consumption between the first and second measurement cycle. Furthermore, the rates for the first measurement cycle were much higher than when the huhu were starved or had an adjustment period (Figure 3.4). Larvae that were starved overnight (12-16 h) and those starved for 24 h had a similar response between the measurement cycles and in their \dot{M}_{O_2} values. In contrast, huhu larvae starved for 48 h had much lower \dot{M}_{O_2} rates.

The final protocol used for subsequent oxygen consumption measurements was selection of actively feeding larvae, based upon the consumption of diet and frass production, starvation overnight in 10 ml syringes, and a 60 min adjustment period after being placed in the respirometry syringes. Data generated using this protocol provided consistent results for huhu of a similar weight (Figure 3.4). There was no statistical difference between larval \dot{M}_{O_2} rates measured during the first and second

cycles ($P = 0.52$). Overnight starvation had the additional advantage of eliminating most of the frass that could potentially block the fine tubes of the respirometers.

Figure 3.4 Mean oxygen consumption rates for different levels of starvation and a 30 min adjustment period, compared with the final protocol of overnight starvation and a 60 min adjustment period. Error bars denote SEM.



Larval size

Larval huhu oxygen consumption at 20°C increased with increasing mass (0.3–3 g) (Figure 3.5). This relationship was highly significant with a good fit for huhu that moved slowly but constantly throughout the measurement period, slope = 0.52 ± 0.07 (95% CI). Lower \dot{M}_{O_2} values were observed to be associated with the small proportion of huhu that were inactive and remained motionless. Respirometry data from neonate larvae, mean weight 1.32 mg, were combined with larger larvae to form a single data set for analysis (Figure 3.6). The slope of the fitted regression line for the larger data set was 0.80 ± 0.015 (95% CI) and was much steeper compared with the narrower weight range of the larger larvae. Neonate larval movement in the respirometry chambers was minimal compared with larger larvae.

Mean larval methane production was 0.028 ± 0.006 ml.kg⁻¹.h⁻¹ (\pm SEM) compared to mean estimated oxygen consumption of the same sized larvae of 170 ± 2.9 ml.kg⁻¹.h⁻¹ (\pm SEM), using the regression equation $\text{Log}_{10} \dot{M}_{O_2} = -0.483 \text{Log}_{10} \text{mass} + 0.489$ and converting $\mu\text{mol.kg}^{-1}.\text{s}^{-1}$ to ml.kg⁻¹.h⁻¹.

Figure 3.5 The relationship between mean huhu larval oxygen consumption and mass at 20°C. The linear regression equation is $\text{Log}_{10} \dot{M}_{O_2} = 0.516 \text{Log}_{10} \text{mass} + 0.489$, $r^2 = 0.73$, $P < 0.0001$, $n = 80$ huhu (95% CI - dotted lines).

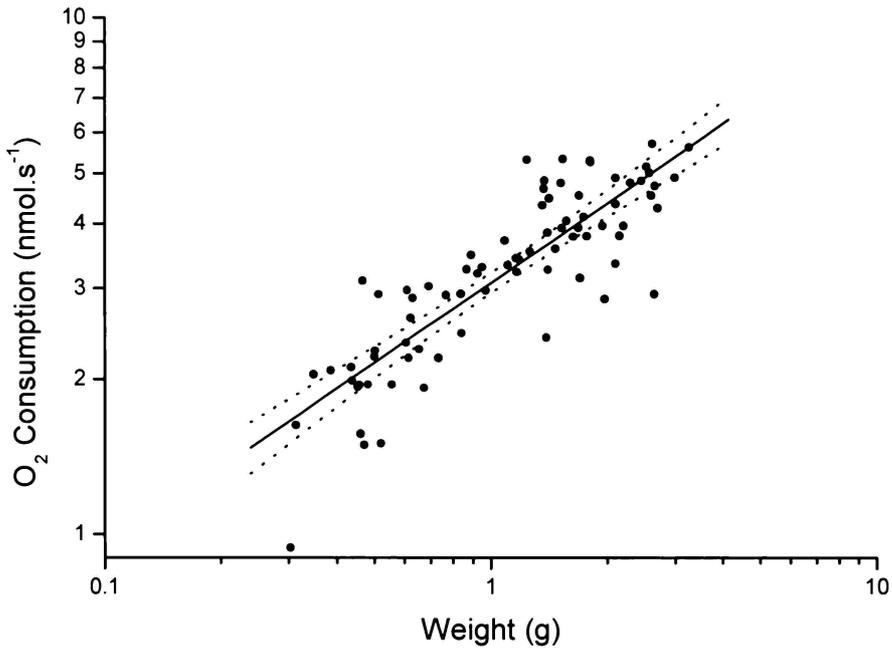
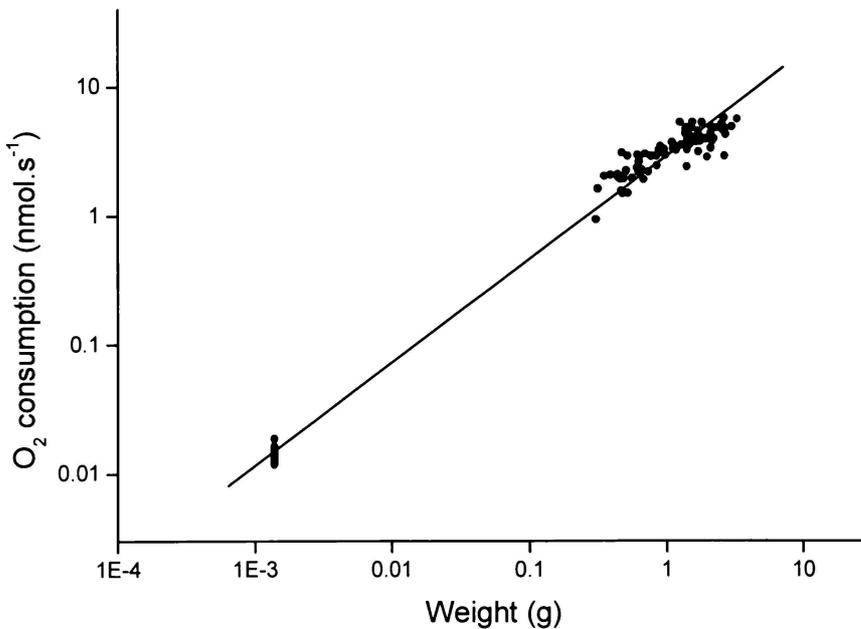


Figure 3.6 The relationship between mean huhu larval oxygen consumption and mass at 20°C, including neonate larvae. The linear regression equation $\text{Log}_{10} \dot{M}_{O_2} = 0.802 \text{Log}_{10} \text{mass} + 0.470$, $r^2 = 0.991$, $P < 0.0001$, $n = 110$ ¹.



¹ $n = 80$ larger larvae plus 30 groups of 10 neonate larvae.

Figure 3.7 displays the same data as Figure 3.5 but oxygen consumption is presented as a specific rate i.e. in $\mu\text{mol.kg}^{-1}.\text{s}^{-1}$ and plotted against huhu larval mass. Presentation of the data in this way illustrates that smaller huhu have a higher metabolic rate per unit mass compared with larger huhu. The mean \dot{M}_{O_2} (specific rate) of neonate huhu was $9.49 \pm 0.18 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$ (\pm SEM; $n = 30$ groups, 10 neonates per measurement group).

Mean \dot{M}_{O_2} (\pm SEM) for larvae exposed to red fluorescent light (dark) was higher than under normal fluorescent light, being 2.84 ± 0.20 ($n = 20$) and $2.46 \pm 0.10 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$ ($n = 45$), respectively, but the differences were not significant ($P = 0.07$).

Pupae

Preliminary oxygen consumption data with pupae of unknown ages were highly variable (D. J. Rogers unpublished data). Therefore pupation dates were recorded to enable oxygen consumption measurements on known age pupae. Pupal age was highly variable due to reliance on field-collected pre-pupae, which restricted the number of insects available for measurement. There was a tendency for \dot{M}_{O_2} to increase with increasing pupal age ($P = 0.002$, Figure 3.8) but the data were highly variable ($r^2 = 0.24$). Male and female pupae both had similar \dot{M}_{O_2} s, despite their weight difference (Table 3.1). Further statistical tests were not performed due to the variability of the data caused by pupal activity.

Table 3.1 Mean oxygen consumption of huhu pupae at 20°C divided into two age groups. Measurements of pupae that moved were excluded (n was the number of pupae measured at each age).

Age (days)	Sex	\dot{M}_{O_2} ($\mu\text{mol.kg}^{-1}.\text{s}^{-1}$) \pm SEM	Weight (g) \pm SEM	n
1-12	♂	1.25 ± 0.11	2.27 ± 0.12	10
	♀	1.19 ± 0.09	2.95 ± 0.13	11
14-24	♂	1.83 ± 0.10	2.24 ± 0.10	8
	♀	1.60 ± 0.11	2.88 ± 0.13	9

Figure 3.7 The relationship between mean huhu larval oxygen consumption per unit mass and mass at 20°C. The linear regression equation is $\text{Log}_{10} \dot{M}_{O_2} = -0.483 \text{Log}_{10} \text{mass} + 0.489$, $r^2 = 0.71$, $P < 0.0001$, $n = 80$ huhu.

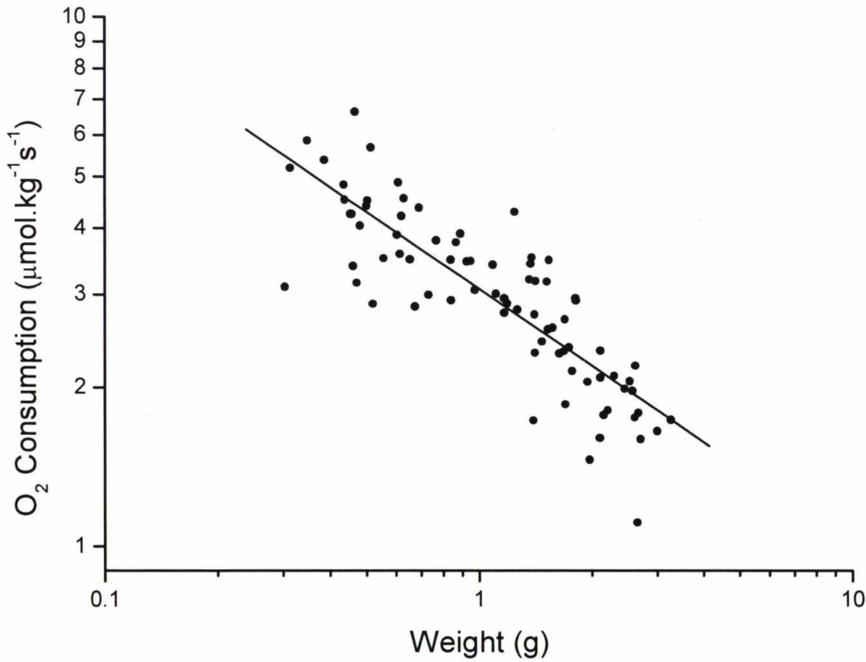
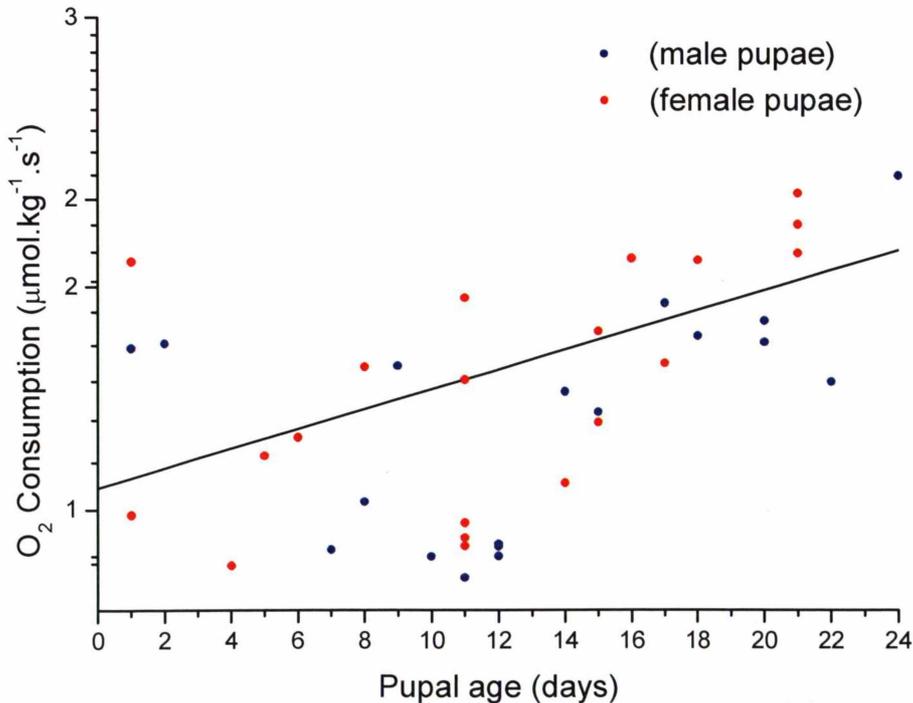


Figure 3.8 Mean oxygen consumption at 20°C of male and female pupae, measured on two occasions 10 days apart. The regression equation is $\text{Log}_{10} \dot{M}_{O_2} = 0.0096 \text{Age} + 0.022$, $P = 0.002$, $r^2 = 0.24$, $n = 38$ ages (22 huhu).



Effect of temperature

Huhu larval oxygen consumption increased with increasing temperature ($P < 0.0001$, Figure 3.9). Data from huhu that moved constantly were excluded from the analysis as movement increased \dot{M}_{O_2} values. At 45°C, \dot{M}_{O_2} declined with elapsed time (Figure 3.10), therefore only the initial values are plotted in Figure 3.9 and 45°C data were excluded from the regression analysis. The mean Q_{10} (the increase in \dot{M}_{O_2} with a 10°C rise in temperature) was 1.72, calculated by taking the antilogarithm of the slope ($0.0237 \pm \text{se } 0.0013$) after first multiplying by 10 (Lighton 1989, Vogt and Appel 1999). The \dot{M}_{O_2} values of larvae exposed to 45°C were initially stable but then steadily decreased with increasing time at this temperature before levelling off again (Figure 3.10). Smaller larvae showed a steeper decline but stabilised at a similar specific value to larger larvae.

The effect of activity

The majority of huhu larvae at 20°C remained active, displaying rhythmic longitudinal and lateral movements during measurements, which therefore represent their active metabolic rate (AMR). The level of activity decreased with increasing temperature until at 35°C there was only slight or no movement (Figure 3.11). At temperatures higher than 35°C, the larvae moved vigorously within seconds of the respirometers being immersed in the water bath and at 45°C all movement ceased after 2-4 min. There appeared to be little difference at 35°C in \dot{M}_{O_2} between larvae that displayed some movement and those that remained immobile. However, at 20°C, mean \dot{M}_{O_2} (\pm SEM) was significantly higher for huhu larvae that constantly moved compared with slightly moving and immobile larvae, AMR = 4.42 ± 0.27 and SMR = $1.66 \pm 0.14 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$, with mean weight 0.52 ± 0.04 g, (ANOVA of Log_{10} transformed \dot{M}_{O_2} , $F = 98$, $P < 0.001$).

Movement in pupae is characterised by rhythmic jerks and also has a dramatic effect on \dot{M}_{O_2} as illustrated for two pupae in Figure 3.12. Activity probably contributed to the variability of pupal \dot{M}_{O_2} even after data from pupae observed moving was discarded, as it was not possible to constantly observe all the pupae with the experimental design used.

Figure 3.9 The relationship between mean oxygen consumption and temperature. The regression equation is $\text{Log}_{10} \dot{M}_{O_2} = 0.024\text{Temperature} - 0.271$, $P < 0.0001$, $r^2 = 0.81$, $n = 84$, error bars denote SEM.

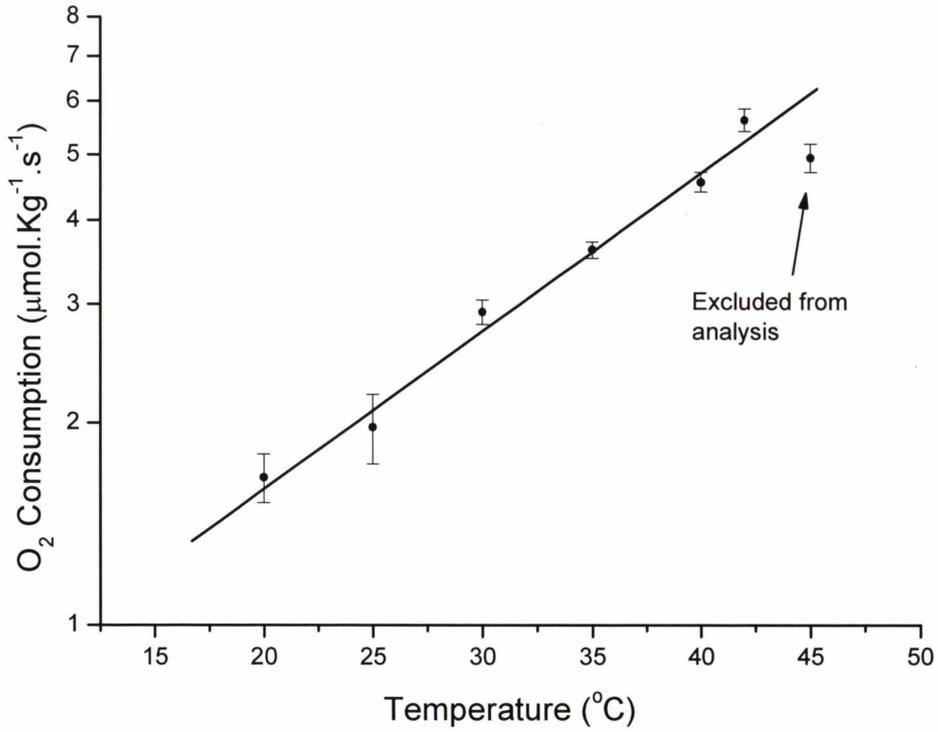


Figure 3.10 Mean oxygen consumption at 45°C of two different sizes of huhu larvae, error bars denote SEM, $n = 6$ larvae for each weight range.

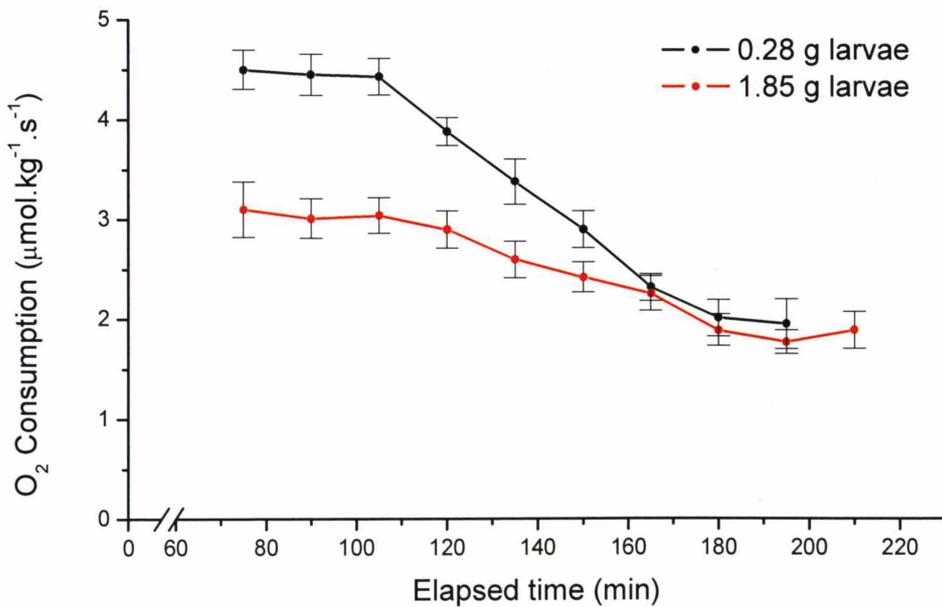


Figure 3.11 The effect of huhu larval activity on mean oxygen consumption at different temperatures expressed as a function of larval weight.

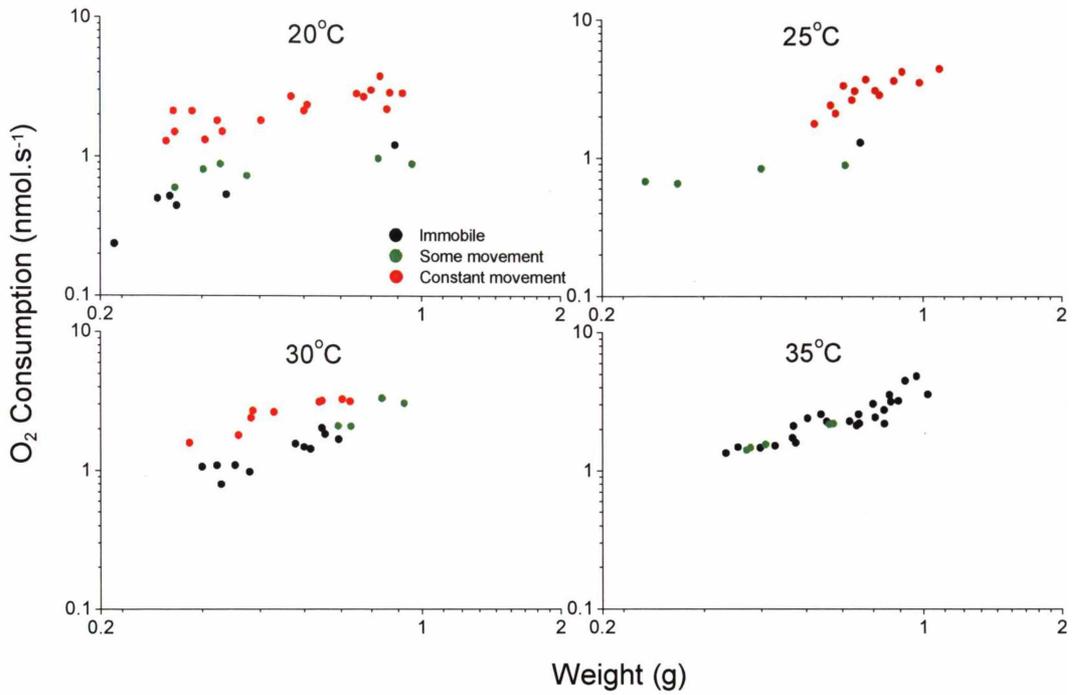
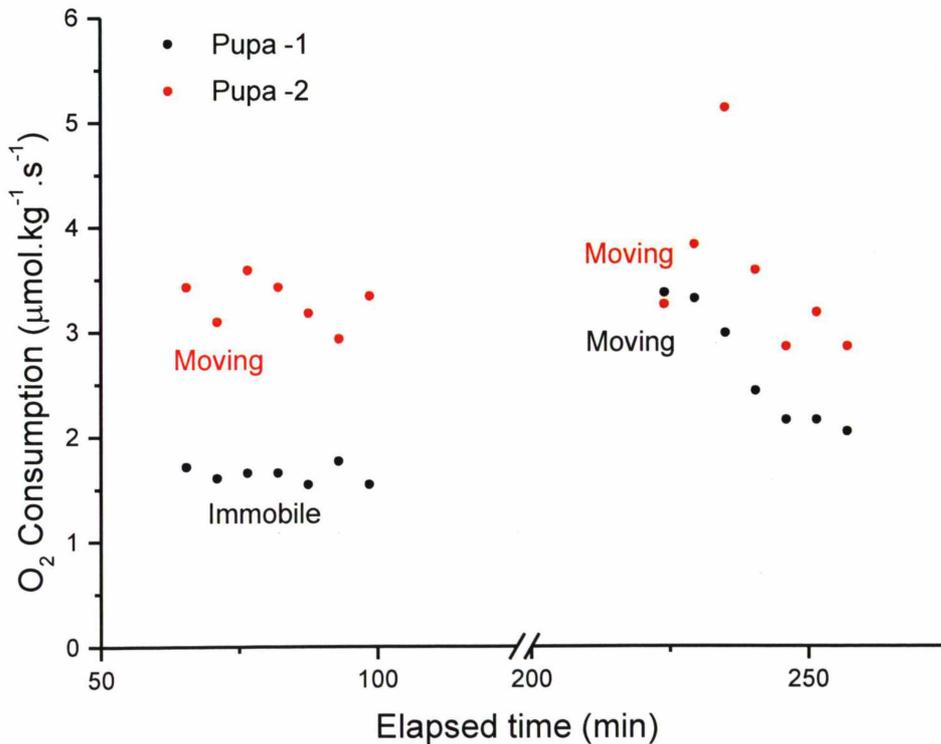


Figure 3.12 Oxygen consumption at 20°C of two female pupae (22 days old), illustrating the effect of activity.



Temperatures within logs

Weekly mean, mean minimum and mean maximum internal log temperatures are shown in Figure 3.13. Daily mean winter (June-August) minimum and maximum temperatures were 8.6 and 11.5°C, respectively (range 2.9 to 18.1°C). Daily mean summer (December-February) minimum and maximum temperatures were 16.1 and 22.1°C, respectively (range 11.5 to 26.8°C). Figure 3.14 shows the difference between air and internal log temperatures and clearly shows the buffering effect of a log on both high and low air temperatures.

Figure 3.13 Weekly mean, mean minimum and mean maximum temperatures recorded within three logs in Riverhead forest, Auckland.

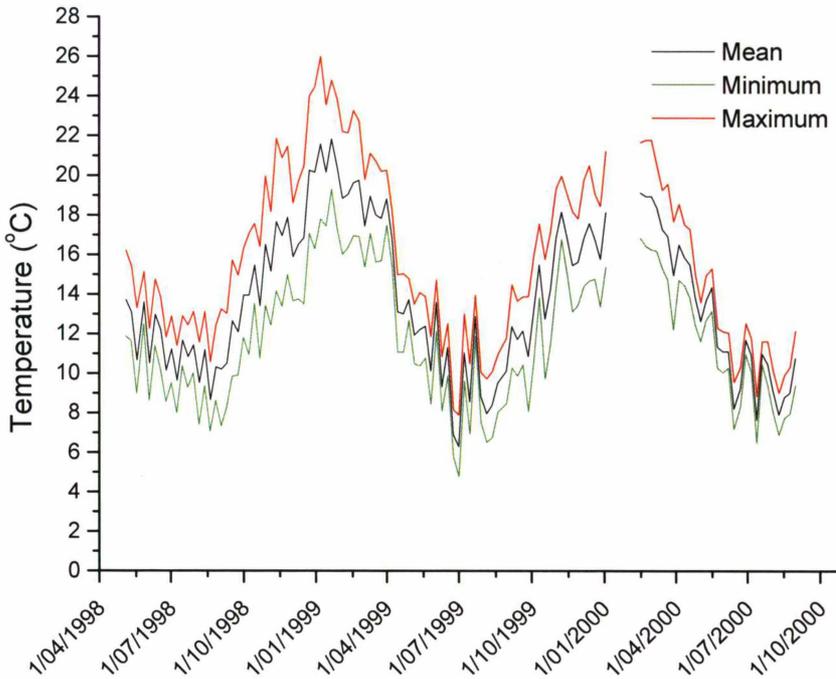
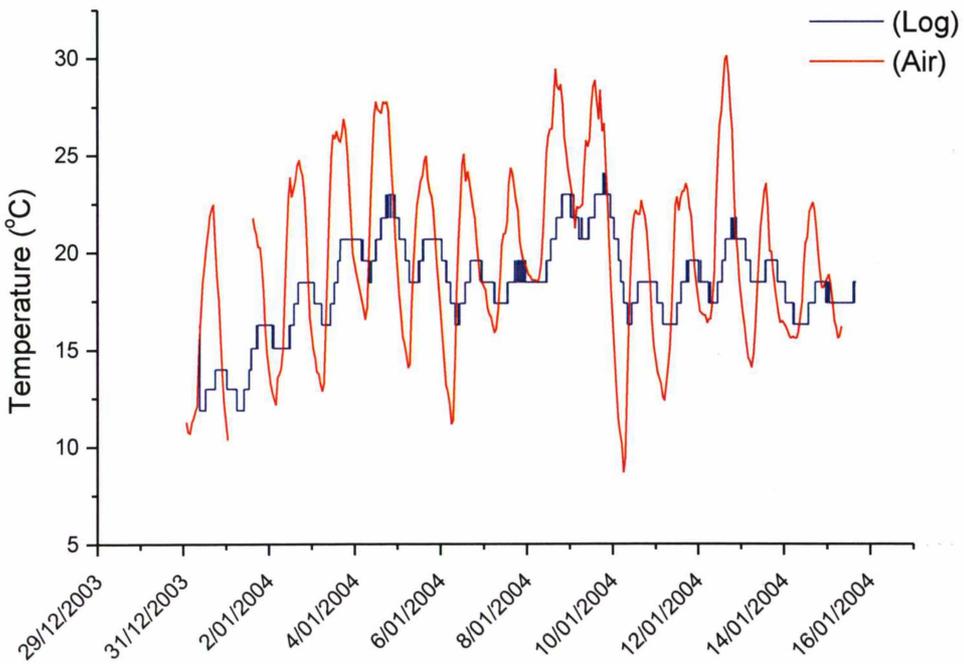


Figure 3.14 Hourly temperature fluctuation within a log under shade compared with air in Hawke's Bay.



Discussion

Measurement

Sampling biases, food deprivation and circadian rhythms can all have a major effect on metabolism yet are not adequately considered by Kleiber's (1961) definition of BMR (Heusner 1971, Lighton 1989, Hayes et al. 1992, Speakman et al. 1993). Speakman (1993) argued that it was illogical and unrealistic to rigidly conform to the Kleiber criteria, instead suggesting that researchers should get as close as possible while considering other uncontrolled factors that affect measurement of metabolism. In this study I have identified several factors that affect SMR and sought to control or measure them.

Recently fed huhu larvae had higher respiration rates consistent with other studies (Lighton 1989, Siegert et al. 1993, Gouveia et al. 2000). Larvae starved for 48 h had noticeably lower rates of oxygen consumption, however, this level of starvation was rejected because it was too far removed from the insects' natural state. Overnight starving was adopted because it provided consistent results and all frass was removed that could potentially block the respirometer. Starvation, while prescribed for measurements of SMR (Kleiber 1961), in small animals can lead to increased activity, reduced body weight and a large reduction in resting metabolic rate (Heusner 1982, Lighton 1989). Starvation in huhu larvae did not lead to these complications, probably due to their visible reserves of fat. Larvae were selected for respirometry that were still actively feeding (prior to starving), thus avoiding insects that had recently moulted or were about to moult and therefore could have lowered metabolism (Siegert et al. 1993). Unfortunately, all pre-measurement conditions failed to prevent larval movement while in the respirometers.

Wood-feeding insects such as termites have symbiotic micro-organisms in their gut that break down cellulose and consequently produce methane and hydrogen (Nunes et al. 1997, Jeeva et al. 1999). Preliminary studies on cerambycids suggest they have cellulase in their guts and probably do not require micro-organisms to digest cellulose (Edwards 1956, Duffy 1963). The artificial diet huhu larvae were fed in this study contained sorbic acid and methyl parahydroxybenzoate to prevent microbial contamination of the diet, but also may have limited gut microorganisms. However, if huhu did produce large amounts of these gases, manometrically-based measurements would have been compromised (Wightman 1977). Fortunately, the volume of methane produced by huhu larvae is insignificant in relation to the

volume of oxygen consumed and therefore would not have interfered with volumetric respirometry. While the test method could not distinguish hydrogen from methane, the latter is more likely. However, neither was present in quantities relevant to this study.

The equipment used had considerable advantages compared to older glass systems (Umbreit et al. 1964). Plastic Luer fittings ensured that leaks were rare, while precise water-bath controllers and micrometer syringes improved the performance of the system. Vibration that probably contributed to insect movement continued to be an issue although was much reduced by changing water baths and installing rubber mountings.

Larval size

The metabolic rate mass-scaling exponent of huhu larvae from 0.3 to 3.27 g was 0.52. This result is lower than Kleiber's (1961) general 0.74 and Heusner's (1982) intraspecific 0.67 value, but huhu larvae continued to move and thus an important aspect of the measurement criteria was not met. When data from neonate larvae, several orders of magnitude smaller, were included in the regression, the slope was 0.80. Metabolic data covering a wide mass range provides more accurate estimates of mass scaling exponents (Schmidt-Neilson 1984). Therefore, the mass exponent of 0.8 may be a better estimate and is more consistent with other studies on insects (Lighton and Fielden 1995, Addo-Bediako et al. 2002). However, there is an absence of data between neonate and larger larvae due to technical issues associated with the sensitivity of the equipment. Regressing $\text{Log}_{10} \dot{M}_{O_2}$ (mass specific rate) on Log_{10} mass simply indicated that small larvae had a higher metabolic rate when expressed per unit mass. The slope was -0.48 compared to the classical rate of -0.25 (Schmidt-Neilson 1984), although when neonate huhu larvae were included the slope was -0.2, which was similar to the classical rate. However, overall larval movement compromised the measurement of the SMR of huhu and hence the relationship to mass.

Pupae

Any observed movements by pupae were recorded and data excluded, but recording volume changes of ten respirometers prevented continuous observation of all movement, which contributed to the variability of the data. Schmolz and Lamprecht (2000) found metabolic heat production during metamorphosis in wax moth followed a standard U-shaped pattern as pupation progressed. They theorised this

related to the two phases of pupation where, initially, larval structures are digested and degraded, followed by the construction of adult tissue. Huhu pupae show an increase in metabolism throughout the pupal period, however, the data are variable with limited observations making further interpretation difficult. The pattern in honey bees is an exception to other holometabolous insects where metabolic rate is initially flat followed by a steady increase until eclosion (Schmolz and Lamprecht 2000). Recording the intermittent rhythmic jerking movements in pupae combined with flow-through respirometry to provide metabolic data in real-time would provide the best estimates of pupal metabolism (Lighton 1991a, Lighton and Fielden 1995).

Effect of temperature

Huhu larval metabolic rate increases with temperature following a similar pattern to other ectotherms (Schmidt-Neilson 1984, Clarke and Fraser 2004). The mean Q_{10} of 1.72 (20-42°C) is close to the average SMR Q_{10} for poikilotherms of 1.67 reported by Robinson et al. (1983) but lower than the 2.0-2.5 discussed by Lighton et al. (2001). Care needs to be taken when comparing published insect Q_{10} values, as they vary depending on life stage, the experimental temperature range, the interpretation of SMR, and latitude (Lighton and Bartholomew 1988, Nielsen et al. 1999, Vogt and Appel 1999, Gillooly et al. 2001). Over a wide temperature range the relationship with metabolism typically displays systematic curvature in many insects. The resulting shape of the Q_{10} response as a function of temperature can be useful for establishing preferred temperature ranges of an animal (Lighton and Bartholomew 1988, Lighton 1989). However, further analysis was not undertaken with the huhu data as it appeared to be linear and at some temperatures the number of observations were limited.

Increased exposure time at 45°C resulted in a declining metabolic rate of huhu larvae, suggesting that the thermal maximum had been exceeded. Exposure to this temperature for 75 min reduced mean \dot{M}_{O_2} well below the regression line for other temperatures. Therefore \dot{M}_{O_2} measurements at 45°C were excluded from the calculation of metabolic Q_{10} . At 35°C most larvae ceased to move and higher temperatures prevented all movement, consistent with the definition of the thermo-stupor point (Salin et al. 1999).

Temperate weather conditions in New Zealand and the buffering effect of logs on the thermal environment result in huhu larvae being exposed to relatively cool

temperatures. Seasonally high temperatures were only experienced for a few weeks and then, usually, only for a small portion of any day. The higher temperatures in Figure 3.13 compared with Figure 3.14 probably resulted from the logs of the former receiving some direct sunlight during part of the day, while the latter were in complete shade. Huhu larvae die when exposed to 25°C for long periods (Rogers et al. 2002); they also fail to develop when timber moisture falls below 25% (Morgan 1960). In their natural environment, huhu larvae almost always occur within shaded logs, which is consistent with a lack of tolerance to heat and sunned logs that dry out too quickly.

Activity

Initial \dot{M}_{O_2} measurements on huhu (larval size) were on larvae that consistently moved within the respirometry chambers. Readings were taken every 5.5 min and showed very little variation within individuals or between larvae, except for a small number of huhu that remained immobile, some of which were observed to moult a few days post-respirometry. Consequently, the initial \dot{M}_{O_2} measurements should be considered as mean active metabolic rates (AMR). Modifications to the equipment that reduced vibration and formal observations of larval activity, as used for the temperature sensitivity experiments, enabled the effect of activity on \dot{M}_{O_2} to be measured. AMR for huhu was 2.7 times SMR, which is consistent with values reported for adult carabid beetles by Chaabane et al. (1999), who also reported AMR values 3-6 times higher than a BMR achieved by anaesthesia with chloroform.

Conclusions

Measuring SMR in huhu larvae and pupae, consistent with published criteria, proved to be difficult (Heusner 1971, Lighton 1991a, Speakman et al. 1993). Constant activity during measurement was the greatest challenge, however, reducing vibration and careful observation enabled the calculation of $SMR = 1.66 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$ for huhu larvae at 20°C with a mean weight of 0.52 g compared to $AMR = 4.42 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$. Activity compromised earlier measurements of SMR on larvae thereby preventing an accurate determination of the effect of size on SMR. The mean temperature sensitivity of huhu larval metabolism was calculated as $Q_{10} = 1.72$ (20-42°C). The natural thermal environment of huhu larvae is buffered by shaded timber, which results in cool temperate conditions.

HUHU RESPIRATION MEASURED USING GAS ANALYSERS

Introduction

Systems for the direct measurement of respiratory gases using optical methods for carbon dioxide and the paramagnetic properties of oxygen have existed for more than 50 years (Kleiber 1961). However, recent advances in gas analyser technology have allowed gas analysers to be used for insect respirometry (Lighton 1991a). Closed system respirometry is analogous to manometric techniques. The insect is confined to a sealed chamber until it alters the component gases, which are measured, and gas exchange rates are calculated based upon the enclosure period. Advantages of systems based upon gas analysers include simultaneous measurement of both oxygen and carbon dioxide, and ease of automation (Lighton 1991a). As the name suggests, open-system respirometry measures the changes the insect makes to the component gases while the gas stream is flowing through the chamber. It is far more demanding on equipment, especially oxygen analysers, and therefore often only carbon dioxide can be measured with very small insects (Lighton 1991a).

Respirometry has been conducted using gas chromatography and with other systems utilising thermal conductivity, but these are often not accurate enough for measurements on insects and are more difficult to automate (Wightman 1977). Calorimetry measures the heat production of an organism and therefore its metabolic rate. Recently, microcalorimetry has become more popular for measuring insect metabolism and is especially useful for developmental studies and different environmental conditions such as hypoxic and hypercapnic atmospheres (Wegener and Moratzky 1995, Schmolz and Lamprecht 2000, Downes et al. 2003). Calorespirometry, which combines respirometry and calorimetry, provides additional information as demonstrated by Acar et al. (2001) who investigated metabolic stress at elevated temperatures in beetles.

Open-system respirometry provides real-time data, enabling easier correlation of insect activity with metabolism, and investigation of an insect's discontinuous gas exchange cycle (DGC) (Lighton 1991a). A typical insect DGC has three phases; a

closed spiracle phase (C), a spiracle fluttering phase (F) and an open spiracle phase (O). Little gas exchange takes place during the C phase, the F phase is primarily oxygen uptake and the O phase releases built-up carbon dioxide (Lighton 1994, 1996). For many years, the dominant theory explaining the evolution of DGCs in insects revolved around water conservation. However, recent publications have suggested that this explanation is rather simplistic, and have proposed that selection for DGCs may have been favoured in hypoxic and hypercapnic environments (Lighton 1994, 1996, 1998, Chappell and Rogowitz 2000). Insect DGCs in tenebrionid beetles and ants have durations of about 6 min but can be as much as several hours in some invertebrates with low metabolic rates (Lighton 1991b, 1994). Measurement of insect metabolic rates based upon carbon dioxide production or respiratory quotient (RQ) values must therefore be at least as long as the insect's DGC (Lighton 1991a).

An animal's RQ, sometimes referred to as the respiratory exchange ratio, is the ratio of oxygen consumption to carbon dioxide production ($\dot{M}_{CO_2} / \dot{M}_{O_2}$), which provides an indication of the substrate used as metabolic fuel. The RQ is typically between 0.7 and 1.0. Lower values are associated with fat metabolism, values of 1.0 and higher with carbohydrate metabolism, while intermediate values are generally either associated with protein metabolism or various mixtures of all three substrates (Kleiber 1961, Schmidt-Neilson 1979). In this study, closed system respirometry was selected due to budgetary constraints, which prevented the purchase of extremely sensitive analysers. Also, as these studies ultimately aimed to examine the effect of extreme temperatures on huhu larvae, measuring an insect's RQ was considered important, and this is often easier with closed systems (Lighton 1991a). Gas analysers were chosen as they can easily be automated and allow calculation of RQ and a wider range of experiments over longer time periods.

The primary objective of this study was:

4. To determine SMR in huhu larvae exposed to 20-40°C and how it is affected by mass and temperature, using automated respirometry.

SMR measurements on huhu at lower temperatures were undertaken to provide data for comparison with responses to stresses caused by extreme temperatures and reduced oxygen atmospheres. A secondary aim was to determine the effect of mass and temperature on huhu larval metabolism. Therefore, the aims were very similar

to those described in Chapter 3, but differ in that they include the use of an automated respirometry system that allowed a greater variety of experiments to be conducted. Many of the components of the automated respirometry system used in these studies were custom built. Consequently, calibration of the components and the system as a whole was vital prior to commencing measurements on huhu larvae.

Materials and methods

Insects

Huhu eggs were collected from felled trees in a commercial pine plantation in Hawke's Bay (Waikoau Forest, Carter Holt Harvey – Forests, New Zealand). Larvae were reared and handled as described in Chapter 3 and Rogers et al. (2002) (Chapter 2). Huhu larvae used for respirometry at 20°C were treated differently, being starved for approximately 20 h with measurements taken between 1600 and 0800 h the following day, L:D 16:8 h (on 0600, off 2200 h). Two size classes of larvae were used; “small” and “large” with weight ranges of 0.5-0.7 g and 3.0-4.0 g, respectively (Table 4.1). Huhu wet weight was used throughout these studies because the size and number of larval instars has not been determined. Additional measurements were made on intermediate weight huhu larvae at 20 and 35°C.

Respirometry

An automated insect respiration system was constructed to measure oxygen consumption and carbon dioxide production in insects. An outline of the automated respirometry system configuration is shown in Figure 4.1. This system was based upon a constant volume respirometry configuration described by Lighton and Bartholomew (1988) and used the integration technique to quantify changes in gas concentrations (Lighton 1991a). The system had 8 chambers and could accommodate 7 insects per respirometry run. Baseline air was supplied from cylinders containing dry air (gas code 108, BOC gases NZ Ltd) with different cylinder oxygen concentrations ranging from 20.7 to 21.18 % and scrubbed of carbon dioxide. Gas flow was regulated with a Flostat MNAB11 controller (Platon, Bassingstoke, UK) prior to passing through an 8-way, micro-inlet manifold fitted with electronically actuated valve assemblies.

Insects were contained in custom-made aluminium and glass respiration chambers connected to the inlet manifold by Tygon[®] tubing (Norton Performance Plastics, Akron, OH, USA). Chambers with an internal volume of either 11.8 or 16.8 ml were used for “small” or “large” huhu larvae, respectively, and could be independently lowered into a temperature controlled water bath. An identical manifold with additional valve assemblies was connected to the downstream outlet of the chambers. A low volume tube containing granular magnesium perchlorate was used to scrub water from the gas stream prior to gas analysis. An infrared carbon dioxide transducer (Servomex 1520/750) followed by a paramagnetic

oxygen transducer (Servomex PM1155B, Sussex, UK) provided measurements of the component gases of interest. A custom built mass flowmeter with a Micro Switch AWM3100 flow sensor (Honeywell Inc., USA) was used to measure excurrent flow rate.

The system was automated by a computerised data acquisition and control system, developed utilising custom-written Labview™ software (Labview™ v. 7.0, National Instruments Corporation, Austin, USA). The method of operation of the system was based upon standard constant volume respirometry systems (Sell et al. 1985, Lighton and Bartholomew 1988, Lighton 1991a). That is, chambers were closed for user-specified times to enable the insects to alter the gas composition; when opened during the analysis cycle the gas concentrations were compared to baseline concentrations from a blank chamber. The fractional oxygen and carbon dioxide concentrations measured by the gas analysers were multiplied by the actual mass flow rate (nominal rate set to 100 standard cubic centimetres per minute, at 0°C and 760 mm of mercury (sccm)) and converted to $\mu\text{l}\cdot\text{s}^{-1}$. Peaks were detected and integrated using the Chromatography Toolkit for Labview™ v. 2.0 (Willstein Software Inc.). Oxygen volumes were corrected for the presence of carbon dioxide using the equation

$$\dot{V}_{O_2} = \frac{\dot{V}i_{O_2} - \dot{V}c_{CO_2} F_{O_2}}{1 - F_{O_2}},$$

where \dot{V}_{O_2} is the rate of oxygen consumption, $\dot{V}i_{O_2}$ is the measured rate of oxygen consumption, $\dot{V}c_{CO_2}$ is the rate of carbon dioxide production and F_{O_2} is the fractional concentration of oxygen in the baseline air (Hill 1972, Withers 1977, Lighton and Bartholomew 1988). Volumes of oxygen depletion and carbon dioxide enrichment were converted to appropriate SI units to yield mass-specific gas exchange rates (wet weight) for oxygen consumption (\dot{M}_{O_2} , $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{s}^{-1}$) and carbon dioxide production (\dot{M}_{CO_2} , $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{s}^{-1}$) (Harrison et al. 1991, Banks et al. 1995). The respiratory quotient (RQ) was calculated and is a dimensionless ratio $\dot{M}_{CO_2} / \dot{M}_{O_2}$. Huhu \dot{M}_{O_2} and \dot{M}_{CO_2} rates were also expressed in $\text{nmol}\cdot\text{s}^{-1}$ when regressed on mass.

The gas analysers were calibrated with oxygen-free nitrogen (zero values), and a standard gas to set the span, prior to each respirometry run (gas code 152 and an alpha standard comprising 21.11 ± 0.02 % oxygen and 4.05 ± 0.02 % carbon dioxide, respectively, BOC Gases NZ Ltd). The signals from both analysers were filtered and amplified using custom-built electronics modules. The oxygen analyser

output was further processed using a zero bias offset amplifier so that small changes in oxygen concentration were effectively amplified from a zero baseline.

Thermistor temperature sensors were fitted to the aluminium ends of two of the respirometry chambers to enable the internal chamber temperatures to be monitored and recorded during each experiment.

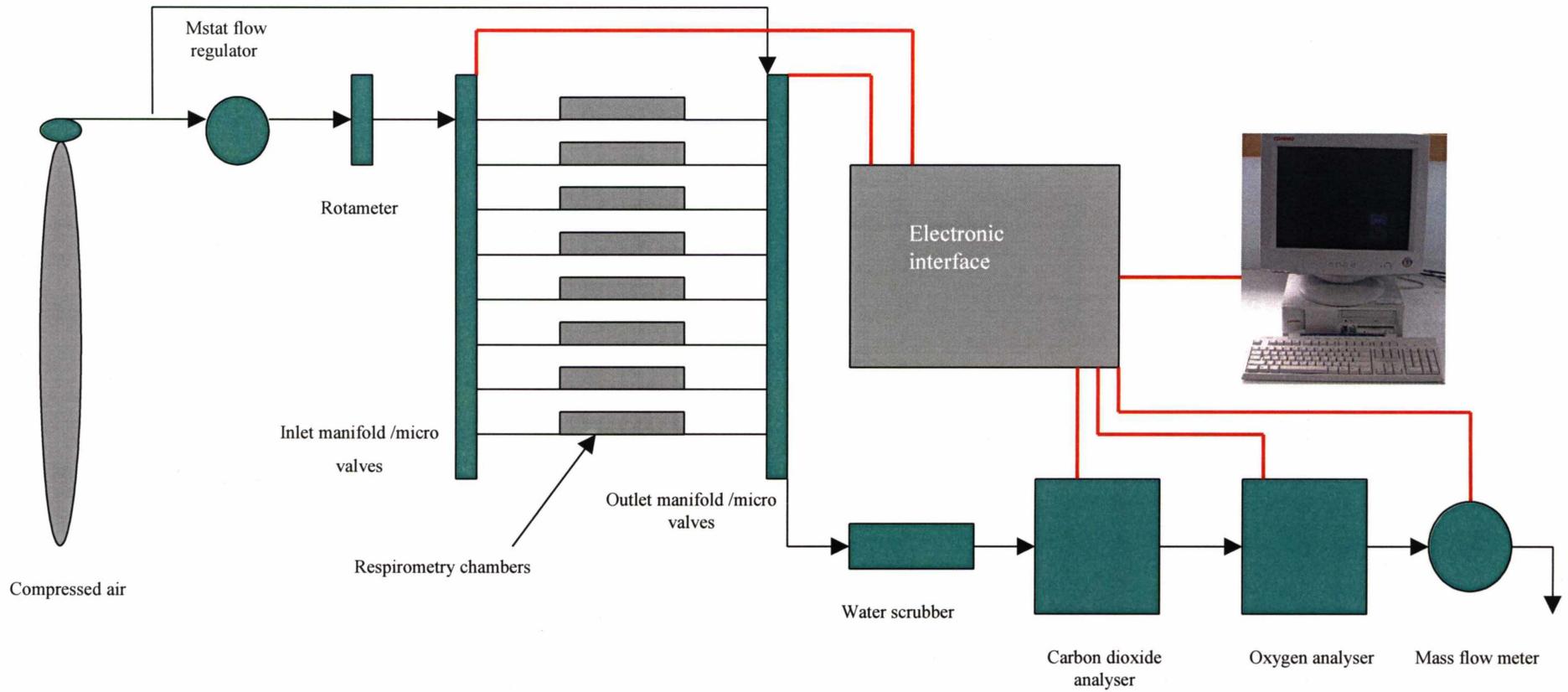
Movement detection

Movement events above preset levels (to remove noise) were measured and recorded using a custom-built movement detection system. Pulsed infrared emitters and detectors were housed in the aluminium ends of each respirometry chamber. Covers made from copper tubing with a small slit to allow observations were slid over the glass respirometry chambers to improve performance by reflecting the infrared light within the chamber. Each detector was standardised by adjusting each chamber's potentiometer without the insect prior to the start of each experiment. When a huhu larva moved within the chamber, less light reached the detector enabling a difference value to be calculated and, ultimately, the activity of that insect. Unfortunately, this apparatus was only available towards the end of the study period. While a system to measure the magnitude of larval movement as well as the number of movements per unit time was originally planned, only the latter was realised. The sums of the movement events were recorded for the same enclosure interval as the gas exchange measurements for each huhu larvae.

Calibration

Reduced oxygen and elevated carbon dioxide gas mixtures were used to calibrate the system. Elevated carbon dioxide levels were generated using gas mixing pumps (Digamix® 300/aF, Wosthoff, Germany) and oxygen depletion generated by injecting nitrogen into the respirometry chambers. Gas mixing pumps were not suitable for generating oxygen-depleted atmospheres for calibration, due to sensitivity of the paramagnetic oxygen analyser to pulsations generated by the pumps.

Figure 4.1 A schematic representation of the automated respirometry system. Air and electrical connections are denoted by the black and red lines respectively.



Also, a β standard gas, comprising $17.8 \pm 0.00\%$ and $2.8 \pm 0.00\%$ oxygen and carbon dioxide, respectively, with the balance nitrogen (BOC Gases Ltd), was used to determine the variability, both between respirometry chambers and measurements, on three different days. The chambers were flushed with the β standard or custom gas mixture via 3-way valves fitted either side of the chamber, then the gas sample was analysed using the same procedures used for huhu larvae. The measured gas volumes were then compared to volumes calculated from the gas concentration and the estimated volume of the respirometry chamber (corrected to STP). The system's mass flow meter was calibrated using a Digital Flow Check-HR™ air flow meter (Alltech Associates, Inc., IL, USA), which was in turn calibrated using Air New Zealand's reference gas flow equipment (Certificate no. 160059610, Calibration Centre, Engineering Services, Air New Zealand, Christchurch, NZ).

Respirometry

Huhu larvae were weighed and sequentially placed in the respirometry chambers at 1.5 min intervals. The system was set to enable airflow through the chambers at about 25 sccm to prevent the atmosphere from being significantly altered by the insect during a 20 min adjustment period, prior to the chambers being automatically closed. Enclosure periods varied depending on the insect mass, chamber size and temperature. Respirometry on huhu larvae was conducted for approximately 5 h, except for larvae at 20°C where the duration was 13 to 16 h, which enabled measurements to be selected from periods of insect inactivity. Details of the measurements carried out on huhu larvae are given in Table 4.1.

To attempt to reduce huhu activity, larvae were exposed to chloroform vapour by placing 400 μ l of chloroform on a small section of dental wick in a 100 ml glass jar at 20°C for 5 min. Larvae were then transferred to the chambers and respirometry carried out at 20°C as described above. However, chloroform anaesthesia was unsuccessful therefore no further larvae were anaesthetised. Instead, measurements from larvae at 20 and 30°C were selected based upon low stable oxygen consumption readings corroborated by direct observations of activity. Oxygen consumption and carbon dioxide production were converted to mass-independent metabolic rates as described by Lighton (2001). Conversions of metabolic rates to alternative units enabled comparisons to be made with published metabolic rates of

other arthropods, and adjustments to different temperatures were made using the formula

$$R_2 = R_1 \cdot Q_{10}^{\frac{T_2 - T_1}{10}}, \text{ which is a rearrangement of } Q_{10} = \left(\frac{R_2}{R_1} \right)^{\frac{10}{T_2 - T_1}}$$

where R_2 and R_1 are the metabolic rates at temperatures T_2 and T_1 and Q_{10} is the change in rate caused by a 10°C increase in temperature (Schmidt-Neilson 1979).

Table 4.1 Exposure temperatures, size, mean mass, number of huhu larvae used, enclosure time and the duration of the experiment.

Temperature ($^\circ\text{C}$)	Size	Mean mass (g) \pm SD	N	Enclosure time (min)	Duration (min)
20	S	0.565 \pm 0.058	21	25	973
	M	1.822 \pm 0.182	21	20-22	864
	L	3.396 \pm 0.299	21	26	793
30	S	0.575 \pm 0.071	21	30	303
	L	3.220 \pm 0.294	21	17	311
35	S	0.770 \pm 0.112	14	30	302
	M1	1.288 \pm 0.131	14	20	298
	M2	1.889 \pm 0.151	14	18	298
	L	3.560 \pm 0.338	21	18	292
40	S	0.641 \pm 0.119	18	25	310
	L	3.593 \pm 0.268	21	12	289

S = Small

M = Medium

L = Large

Statistics

Gas exchange data were analysed using a residual maximum likelihood (REML) function on Log_{10} transformed response data using a linear mixed model. REML is especially suitable for moderately unbalanced designs as it weights each response value to reflect the variance contributions from different sources. Consequently the means obtained using REML are referred to as estimated means, and will only be equal to the arithmetic mean if the design is balanced. Larval mass and temperature terms were used for the fixed model and individual huhu for the random model.

The Wald statistic was used to test the significance of the fixed effects terms as they were added to the model. The Wald statistic is the ratio of the square of the estimated parameter value to the curvature of the likelihood function. It has an asymptotic χ^2 distribution with degrees of freedom equal to that of the parameter. If the design is balanced the Wald statistic divided by its degrees of freedom will be distributed as $F_{n,m}$, where m is the degrees of freedom of the fixed effect and n is the number of residual degrees of freedom of the fixed effect. RQ data were analysed using one-way analysis of variance (ANOVA), with mass as a covariate to examine the effect of temperature. Means were compared for significance ($\alpha = 0.05$) using Tukey's HSD. Genstat (Genstat V.7.2, VSN International Ltd, UK) was used for the statistical analysis described above. Regression lines were fitted and plotted using Origin™ (Version 7.5, Microcal Software Inc., MA, USA).

Results

Calibration

The slopes and r^2 values of the regressions of measured oxygen depletion and carbon dioxide enrichment on calculated amounts indicate the analysis system accurately measured these gases (Figure 4.2 and 4.3). Furthermore the response was linear and repeatable. One way ANOVA of the percentage difference of measured to calculated values demonstrated there were no significant differences between oxygen measurements on different days (with a new calibration to zero and span gases on each day), $P = 0.07$, $F_{2, 39} = 2.89$ $n = 42$ readings. There were, however, significant differences between chambers, $P < 0.001$, $F_{6, 35} = 6.01$, with chamber 7 being consistently lower than the others. However, the magnitude of the percentage difference was small, the overall mean difference was $-3.0 \pm 0.17\%$ and chamber 7 was $-4.6 \pm 0.26\%$. Therefore this difference of 1.6% was ignored, as it was small compared to biological variation.

Figure 4.2 Mean measured oxygen volumes compared with calculated (\pm SEM). The regression equation is $O_2 \text{ measured} = 1.03 \times O_2 \text{ calculated} - 0.45$, $r^2 = 0.99$ (95% CI - dotted lines).

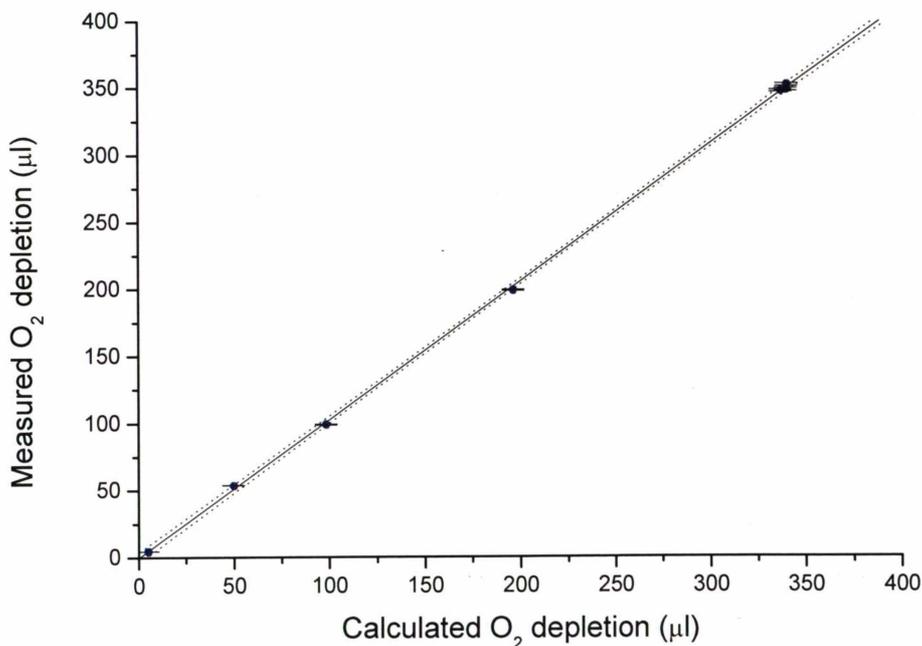
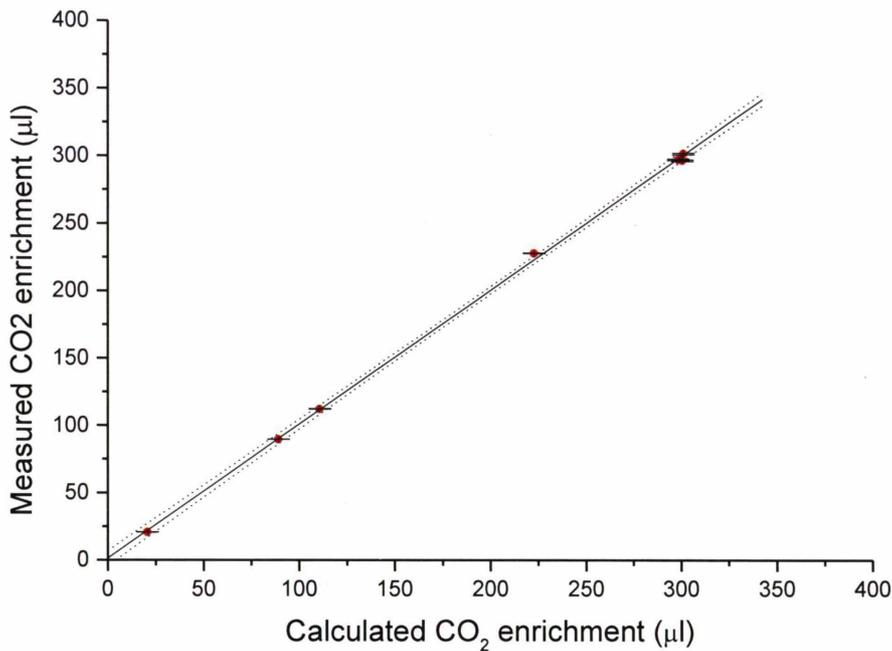


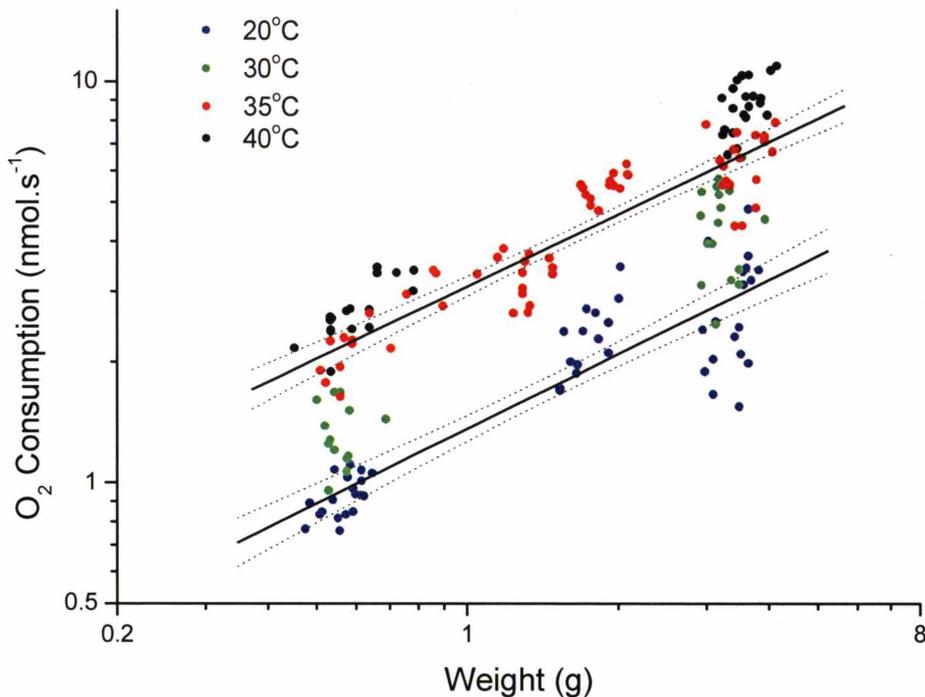
Figure 4.3 Mean measured carbon dioxide volumes compared with calculated (\pm SEM). The regression equation is $\text{CO}_2 \text{ measured} = 1.00 \times \text{CO}_2 \text{ calculated} + 1.33$, $r^2 = 0.99$ (95% CI - dotted lines).



Effect of mass and temperature

Regressions of inactive huhu larval oxygen consumption on mass at 20 and 35°C had similar slopes of 0.621 ± 0.043 and 0.603 ± 0.034 , respectively (\pm SE) (Figure 4.4). Furthermore, data at 30 and 40°C displayed a similar pattern showing the effect of mass scaling and temperature on huhu metabolism. Carbon dioxide production followed a similar pattern and therefore has not been depicted graphically. Analysis of the complete dataset demonstrated that both temperature and mass were significant determinants of oxygen consumption and carbon dioxide production, however, the interaction of mass and temperature was not significant (Table 4.2). Therefore, gas exchange in huhu larvae can be described by the simple equations listed in Table 4.2. The parameter estimates for mass, i.e. the common slopes, were 0.62 ± 0.033 and 0.67 ± 0.038 (\pm SE), which were the mass-scaling exponents (see Chapter 3) for huhu larvae (20-40°C) of oxygen consumption and carbon dioxide production respectively (Lighton and Duncan 1995).

Figure 4.4 The relationship between mean oxygen consumption and mass at 20 and 35°C for inactive huhu larvae. The linear regression equations for 20 and 35°C respectively are $\text{Log}_{10} \dot{M}_{O_2} = 0.621\text{Log}_{10}\text{mass} + 0.136$, $r^2 = 0.81$, $P < 0.0001$, $n = 51$ and $\text{Log}_{10} \dot{M}_{O_2} = 0.603\text{Log}_{10}\text{mass} + 0.492$, $r^2 = 0.84$, $P < 0.0001$, $n = 63$ (dotted lines indicate the 95% CI of the line).



Huhu \dot{M}_{O_2} data were converted to mass-independent values by dividing by the larval mass^{0.62}; 0.62 being the mass-scaling exponent for this temperature range and then regressed on temperature (Figure 4.5) (Schmidt-Neilson 1984, Lighton et al. 2001). The calculated mean Q_{10} from the slope was 1.69 (based on slope = $0.02283 \pm \text{SE } 8.06\text{E-}4$) and determined as described in Chapter 3. Huhu \dot{M}_{CO_2} data were also converted to mass-independent values using the mass-scaling exponent 0.67 and regressed on temperature to give a mean Q_{10} of 1.90 (based on slope = $0.02789 \pm \text{SE } 9.06\text{E-}4$). Therefore, the temperature dependence of \dot{M}_{O_2} and \dot{M}_{CO_2} are different. To further explore the thermosensitivity of the Q_{10} values, a cubic polynomial was fitted to the oxygen and carbon dioxide temperature data (Lighton 1989). The polynomial equations were $\text{Log}_{10} O_2 = -1.66039 + 0.15746t + -0.00425t^2 + 4.28477\text{E-}5t^3$ and $\text{Log}_{10} CO_2 = -1.24337 + 0.09147t + -0.00182t^2 + 1.61359\text{E-}5t^3 \text{ nmol.s}^{-1}$, where $t = \text{temperature}$, $P < 0.001$, $n = 185$, r^2 was 0.82 and 0.84 respectively. Predicted Q_{10} values were calculated from the polynomial equations for the temperature ranges of interest using the standard Q_{10} formula (see

methods). Calculated Q_{10} values decreased to a minimum between 30 and 35°C for oxygen consumption prior to increasing, while carbon dioxide production values declined with increasing temperature (Table 4.3).

Analysis of the actual RQ data (not calculated from the mass-independent values) shown in Table 4.4 confirms this difference in thermosensitivity. One-way ANOVA with mass as a covariate demonstrated temperature significantly increased RQ ($F_{3, 180} = 102, P < 0.001$). To remove the effect of mass, means were adjusted for the covariate and compared using Tukey's HSD, which demonstrated that all temperatures resulted in significant differences in larval RQ values except 35-40°C (Table 4.4).

Table 4.2 The significance and relationship of mass and temperature (20-40°C) as determinants of gas exchange in huhu larvae. Errors denote the SE of the slope.

	Fixed term	Wald statistic	d.f.	Wald/d.f.	χ^2 Prob.
Oxygen consumption	Temperature	973	3	325	< 0.001
	Mass	1306	1	1306	< 0.001
	Temp.Mass	2.49	3	0.83	0.478
Carbon dioxide production	Temperature	1101	3	367	< 0.001
	Mass	1111	1	1111	< 0.001
	Temp.Mass	0.83	3	0.28	0.841

$$\text{Log}_{10}\text{O}_2 = A + 0.624 \pm 0.033\text{Log}_{10}\text{Mass}$$

$$\text{Log}_{10}\text{CO}_2 = A + 0.668 \pm 0.038\text{Log}_{10}\text{Mass}$$

Temperature	Constant A for O ₂	Constant A for CO ₂
20°C	0.256	0.117
30°C	0.521	0.429
35°C	0.609	0.552
40°C	0.707	0.667
SED (average)	0.018	0.022

Figure 4.5 The relationship between huhu larval mass-independent mean oxygen consumption and mean carbon dioxide production, and temperature. The regression equations are $\text{Log}_{10} \dot{M}_{O_2} = 0.02283\text{Temperature} - 0.312$ ($P < 0.0001$, $r^2 \text{ adj} = 0.81$) and $\text{Log}_{10} \dot{M}_{CO_2} = 0.02789\text{Temperature} - 0.561$ ($P < 0.0001$, $r^2 \text{ adj} = 0.84$), $n = 185$. Error bars denote SEM.

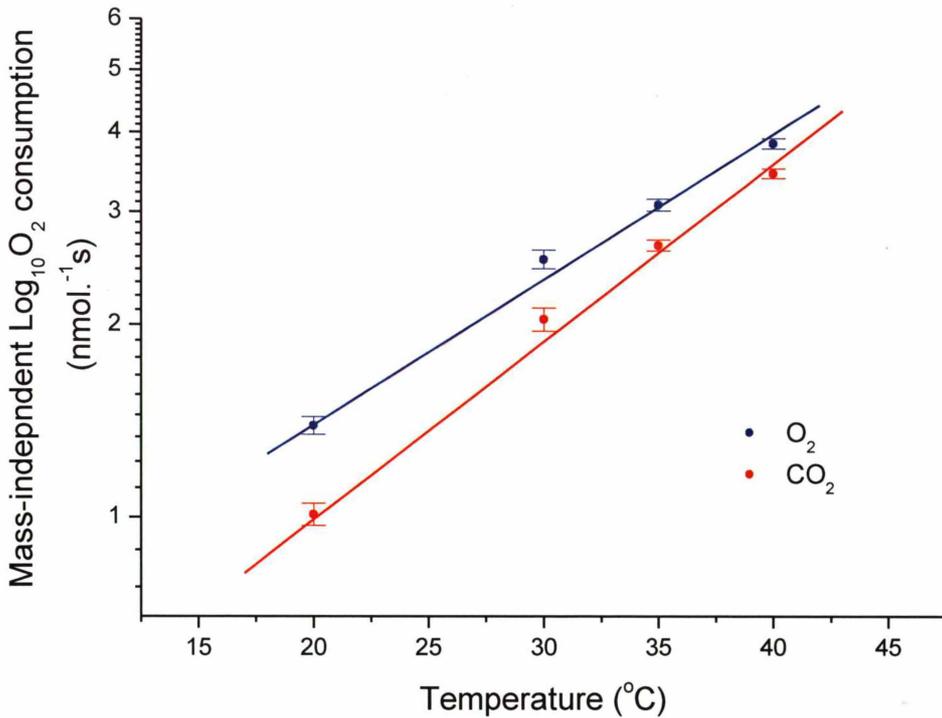


Table 4.3 Predicted Q_{10} values for different temperature ranges of mass-independent oxygen consumption and carbon dioxide production of huhu larvae.

Temperature range (°C)	Q_{10}			
	20-25	25-30	30-35	35-40
O ₂ consumption	2.07	1.63	1.49	1.58
CO ₂ production	2.20	1.91	1.75	1.70

Table 4.4 Mean huhu larval RQ at elevated temperatures, adjusted for the covariate (weight). Means not followed by the same letter are significantly different, $P < 0.05$, Tukey's HSD.

	Temperature (°C)			
	20	30	35	40
RQ	0.73	0.81	0.88	0.91
	a	b	c	c

SED 0.012, $P < 0.05$

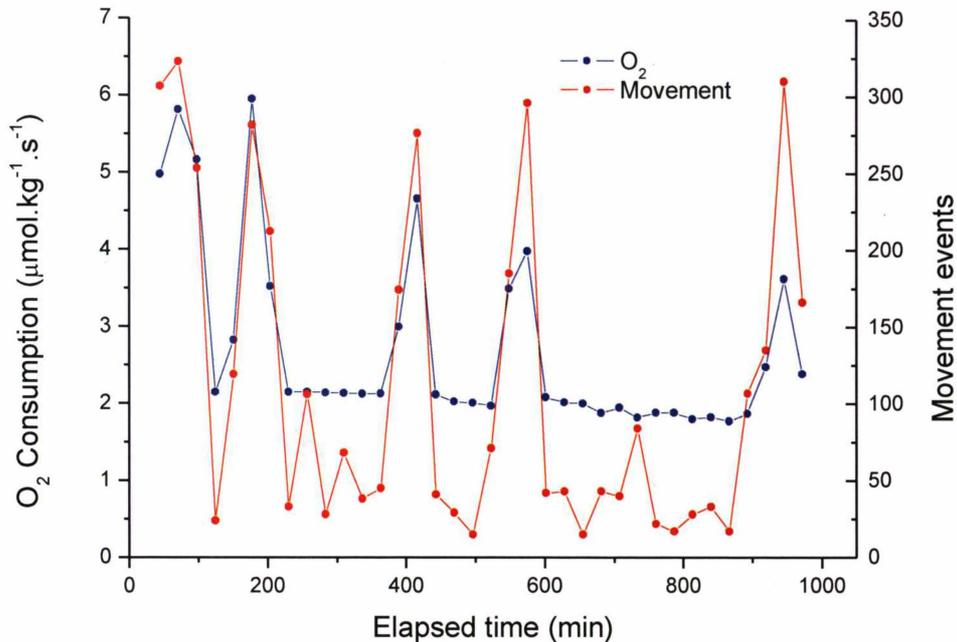
The effect of activity

Increased time in the respirometry chambers at 20°C and reduced vibration by isolating the chamber support mechanism from the water bath resulted in most huhu larvae having periods of inactivity. Inactivity was easily identified by low consistent oxygen readings that were confirmed by observing larval activity. Data from a typical small larva is shown in Figure 4.6. This pattern was confirmed by activity measurements that followed a very similar pattern to oxygen consumption. Unfortunately the movement detection system was only available for a small number of measurements towards the end of the investigations. Figure 4.6 also illustrates that activity resulted in substantial increases in oxygen consumption; in this case up to three times as much as the SMR. However, slight movement above the preset threshold did not appear to affect \dot{M}_{O_2} . Oxygen consumption data plotted against movement for the same huhu larva are shown in Figure 4.7 and indicate an expected sigmoidal type increase. However, when data from seven huhu were examined the pattern appeared to be more linear with some variability (Figure 4.8). It was not possible to determine if this variability was due to design issues or erroneous measurements. RQ values of huhu larvae appeared to be unaffected by movement (Figure 4.8). Regression of oxygen consumption on activity for individual huhu provided an estimate of \dot{M}_{O_2} at movement = 0, i.e. the intercept. Mean \dot{M}_{O_2} values calculated using this method compared with means from stable readings were 1.80 ± 0.067 and $1.77 \pm 0.078 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$, respectively (\pm SEM, $n = 6$ small huhu).

After exposure to chloroform, larval activity was noticeably reduced in magnitude and duration, and mean \dot{M}_{O_2} was similar to inactive larvae. However, huhu treated

with chloroform still displayed some movement and had an initial mean (\pm SEM) RQ of 0.56 ± 0.01 (45 min), which steadily increased to 0.84 ± 0.03 after 310 min.

Figure 4.6 Oxygen consumption and movement data, typical of a small huhu larva. Movement data was recorded as the number of movement events occurring during the enclosure period for the same oxygen reading.



Standard metabolic rate

The mean mass-independent SMR for huhu larvae exposed to 20 and 30°C and converted to 25°C (using the mean Q_{10} of 1.69) and mass-independent units using the mass exponent of 0.62 was 1.88 ± 0.047 nmol.s⁻¹ (\pm SEM, $n = 79$). The pattern of carbon dioxide production of some large huhu larvae was irregular despite remaining motionless and the oxygen consumption remaining stable. A typical example is shown in Figure 4.9. However, calculations of \dot{M}_{CO_2} were not affected, as this pattern of ventilation was associated with longer periods of inactivity and \dot{M}_{CO_2} was averaged for each insect.

Figure 4.7 The relationship between oxygen consumption and movement events per period of a small huhu larva (the same larva as Figure 4.6).

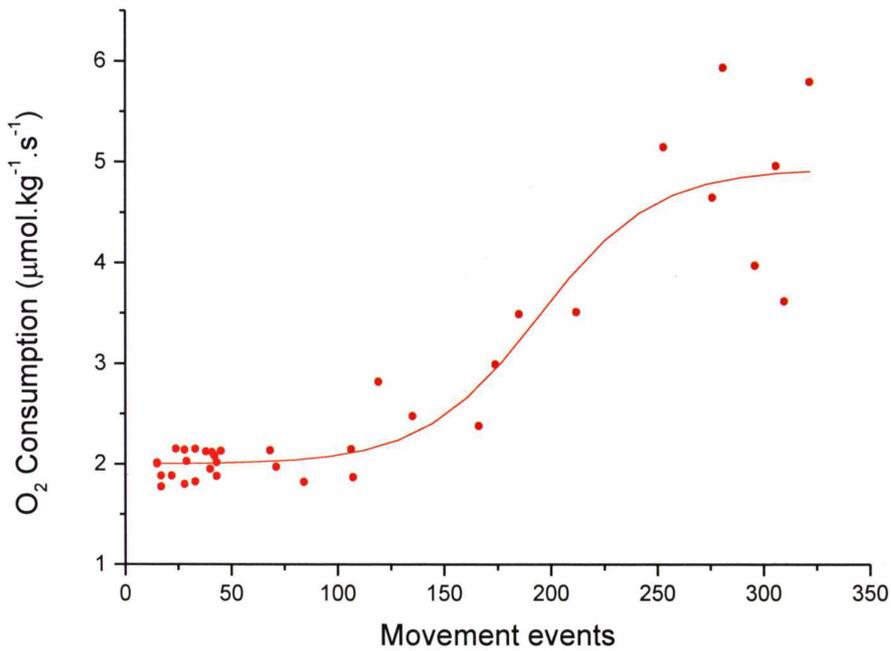


Figure 4.8 The relationship of movement events per period to oxygen consumption and RQ, $n = 7$ small huhu larvae.

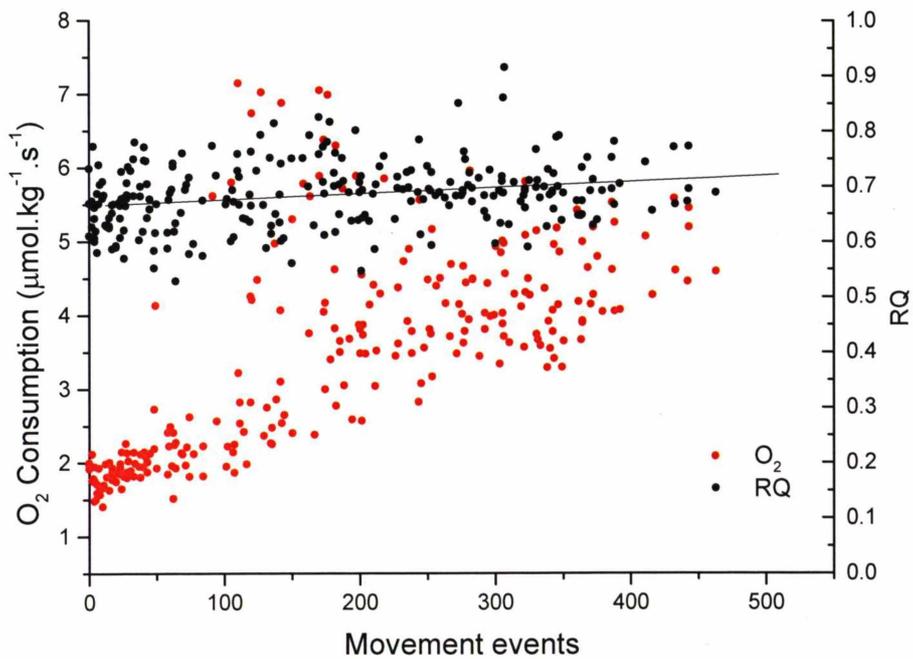
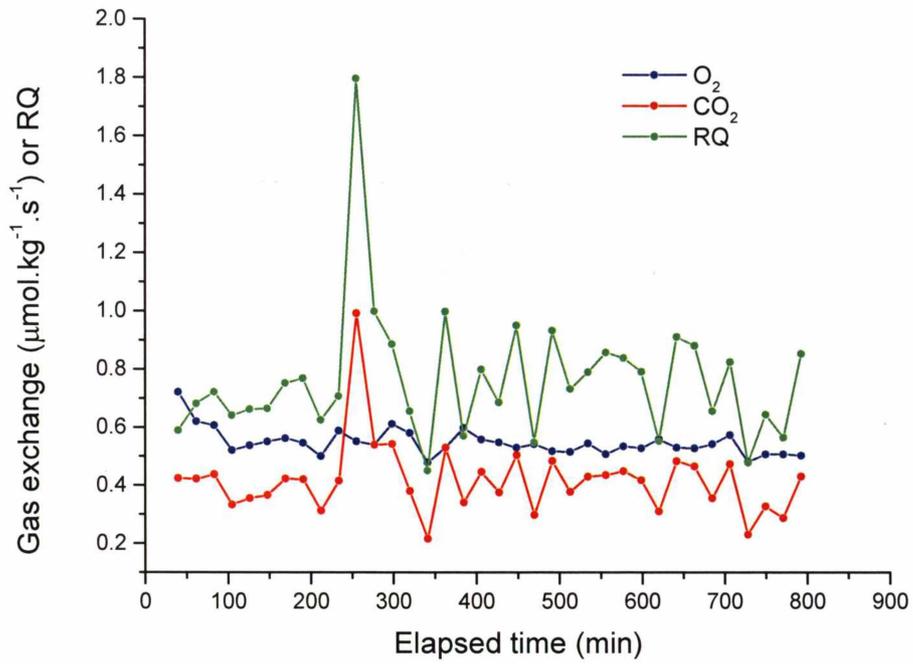


Figure 4.9 Oxygen consumption, carbon dioxide production and RQ for a large huhu larva. Note the irregular pattern of carbon dioxide production.



Discussion

Calibration

Calibration of any scientific instrument is vitally important to ensure accurate and repeatable measurements. For example, a 5% error in measured air mass-flow during respirometry using the integration method, results in the same percentage error in measured \dot{M}_{O_2} and \dot{M}_{CO_2} . In this study, thermometers, air mass-flow meters and standard gases were all traceable to a recognised standard. The paramagnetic oxygen and infrared carbon dioxide transducers and mass-flow sensor were all factory-made, however, all the electronics associated with these sensors were custom designed and built. Therefore it was necessary to determine if the entire gas analysis system could accurately measure changes in gas concentrations. Regression of measured oxygen depletion and carbon dioxide production on calculated values demonstrated the system was able to accurately measure changes in gas composition of the magnitude caused by huhu larvae.

Effect of mass

The mass-scaling exponent for huhu larvae of 0.62 (20 - 40°C, based on oxygen consumption) was close to the within-species value of 0.67 as discussed by Heusner (1982). Furthermore, when the mass-scaling exponent was calculated from carbon dioxide data the value was 0.67 and was not significantly different from the value calculated using oxygen data. Lighton and Fielden (1995) calculated an interspecific mass-scaling factor of 0.825 based upon tenebrionid beetles and formicine ants. Analysis of published metabolic rates of 346 insect species enabled Addo-Bediako et al. (2002) to estimate the factor at 0.768. However, both studies only considered adult insects and calculated interspecific mass-scaling exponents. Furthermore, mass-scaling of metabolism has always been highly controversial with discussion about whether 0.75 as proposed by Kleiber (1961) as an interspecific mass scaling exponent may be a statistical artefact (Heusner 1982, Feldman and McMahon 1983). Also, the method used to calculate SMR (open or closed respirometry) and whether the species is capable of flight, can affect results (Lighton and Fielden 1995, Reinhold 1999, Lighton et al. 2001, Addo-Bediako et al. 2002). The mass-scaling exponent of 0.52 reported in Chapter 3 was compromised by movement and was considerably lower than that obtained here. Activity levels of huhu larvae in this study were carefully monitored and comparatively large numbers of insects were measured over an extended period, therefore leading to a more accurate measurement of the mass-scaling exponent.

The effect of temperature

Huhu metabolism, as measured by both oxygen consumption and carbon dioxide production, increased in response to increasing temperature within the range of 20-40°C. The mean Q_{10} of 1.69 calculated using mass-independent oxygen consumption data was not significantly different from the Q_{10} of 1.72, obtained using manometric methods described in Chapter 3 using a much smaller data set. Q_{10} is a useful concept to describe the biological effects of temperature but is not supported by thermodynamic theory and actually varies with temperature (Schmidt-Neilson 1979, Gillooly et al. 2001). Figure 4.5 indicated huhu metabolic responses to temperature were probably not linear when plotted on semi-log scale. Therefore a cubic polynomial was fitted to calculate the thermosensitivity of Q_{10} as described by (Lighton 1989). However, the differentiation method described by Lighton (1989) was not used, based upon advice from a statistician. The predicted gas exchange values from the polynomial equations were used to calculate Q_{10} , using the standard formula described in the methods. The Q_{10} values based on both \dot{M}_{O_2} and \dot{M}_{CO_2} vary with temperature, indicating that the thermo-sensitivity of metabolism is not linear, especially over wider temperature ranges. Q_{10} values are often calculated from carbon dioxide production data, especially when open system respirometry is used on small insects. In this study the Q_{10} s calculated for \dot{M}_{CO_2} were considerably different to those for \dot{M}_{O_2} , for both the mean and individual values at different temperatures. Therefore, researchers should ideally measure the temperature sensitivity of both \dot{M}_{O_2} and \dot{M}_{CO_2} .

The RQ at 20°C of 0.73 suggested primarily fat metabolism, but in huhu larvae the RQ increased with temperature, suggesting fat metabolism was being supplemented by other substrates such as carbohydrate and or protein (Schmidt-Neilson 1979). Extensive studies on the effect of temperature, dietary sucrose concentration and sorbitol synthesis in silverleaf whitefly respiration demonstrated an apparent requirement of higher dietary concentrations of sucrose at temperatures greater than 30°C (Salvucci et al. 1999, Salvucci and Crafts-Brandner 2000). Furthermore, in this insect, high temperature appeared to alter metabolic activity that led to increased availability of fructose and stimulation of the pentose-phosphate pathway. In huhu it would clearly be advantageous to respond rapidly to greater metabolic demand at high temperatures by supplementing fat metabolism with carbohydrate reserves. The two most commonly stored insect carbohydrates are trehalose and glycogen, the former being present in large quantities in haemolymph, where it can

be rapidly hydrolysed into two glucose molecules (Nation 2002). Many researchers assume RQ stays constant across a range of temperatures, which with huhu larvae would have introduced considerable error.

Measurements of gas exchange were used in this study to indicate changes in metabolic rate. If both oxygen consumption and carbon dioxide production have been measured the relationship is

$$MR = (15.97 + 5.164 RQ) \dot{V}_{O_2}$$

where MR is the metabolic rate (J s^{-1}), RQ is the respiratory quotient, and \dot{V}_{O_2} is the oxygen consumption ($\text{cm}^3 \text{s}^{-1}$) (Lighton 1991a). Consequently, MR depends upon the RQ and hence the metabolic substrate. Therefore, because the RQ for huhu changes from 0.73 at 20°C to 0.91 at 40°C, the increase in oxygen consumption would not be equivalent to the increase in MR. Indeed, MR would have a greater increase with temperature compared with oxygen consumption. The Q_{10} for huhu based upon oxygen consumption is still correct but would be not equivalent to the thermo-sensitivity of metabolism.

Activity

Activity in the respirometry chambers continued to be an issue, although reducing vibration and longer periods in the chambers resulted in consistent measurements on inactive larvae. Other researchers have had similar problems obtaining standardised measurements from insects and have attempted to solve them using such invasive methods as anaesthesia, decapitation, restraint, reducing vibration and increased starvation (Lighton and Garrigan 1995, Neven 1998, Chaabane et al. 1999). An alternative approach is to use movement detection and either eliminate data associated with movement or use the relationship between activity and metabolism to calculate resting rates (Heusner 1971, Lighton and Feener 1989, Lighton and Fielden 1995). Closed respirometry is more susceptible to erroneous measurement due to increased activity because of the poor temporal resolution imposed by the method (Lighton and Fielden 1995). In this study, enclosure periods were minimised by using relatively small chambers allowing better identification of inactive periods. By making careful observations of activity it was established that low consistent oxygen consumption readings were the best measure of inactivity. Chaabane et al. (1999) used chloroform to anaesthetise carabid beetles to obtain a basal metabolic rate. While chloroform reduced activity and provided similar

\dot{M}_{O_2} measurements to inactive huhu larvae, \dot{M}_{CO_2} and hence RQ was very different suggesting chloroform was not suitable for huhu.

Data from huhu larvae that moved were excluded from analysis by selecting consistent stable readings. Measurement of activity using a movement detection system for 6 small larvae and using the relationship between movement events and \dot{M}_{O_2} provided similar results to selecting stable readings, which confirmed the effect of activity on metabolism. However, as the data were a repeated measure on the same individual, an assumption of regression, (i.e. independence) was not met. This design also applies to the combined data in Figure 4.7, therefore no further analysis was undertaken, but the pattern of the data suggests RQ did not vary with activity.

The movement detection system required more development to be fully operational. One limitation was that it operated along the axis of the respirometry chamber allowing larvae that were close to the emitter or detector to generate different data than if occupying the middle of the tube. Also it did not effectively measure the magnitude of movement, which may contribute to some of the high \dot{M}_{O_2} measurements associated with low activity shown in Figure 4.7.

Standard metabolic rate

Reinhold (1999) proposed a general hypothesis to explain interspecific differences in size-independent resting metabolic rate (RMR), which was based on a trade-off between low RMR and adaptations of metabolism during activity. Therefore he categorised published insect RMRs based upon the ability to fly, and postulated that low RMR would be associated with non-fliers. This categorisation explained 64% of the variance in RMR that was not explained by body mass. Huhu larval SMR, converted to the same units, was $0.144 \pm 0.0035 \text{ ml O}_2 \text{ g}^{-0.75} \cdot \text{h}^{-1}$ (\pm SEM, $n = 79$) and placed it amongst the non-fliers with low RMR. For example, coleopterans that flew had RMRs ranging from 0.255 to 1.430 $\text{ml O}_2 \text{ g}^{-0.75} \cdot \text{h}^{-1}$, whereas those that were non-fliers ranged from 0.135 to 0.286 $\text{O}_2 \text{ g}^{-0.75} \cdot \text{h}^{-1}$ (Reinhold 1999). Lighton et al. (2001) has proposed a non-tick, non-scorpion, non-flying, general arthropod equation of:

$$\text{MR} = 973M_b^{0.856},$$

where MR is the metabolic rate in μW and M_b is body mass in g at 25°C . Using this equation the estimated SMR for a 1g insect is $973 \mu\text{W}$, which converts to $180 \mu\text{l O}_2\text{h}^{-1}$. Huhu larval data from exposure to 20 and 30°C , converted to 25°C (using the mean Q_{10} of 1.69) and 1 g mass using the mass exponent of 0.62 is $152 \pm 0.0038 \mu\text{l O}_2\text{h}^{-1}$ (\pm SEM, $n = 79$). Therefore huhu larvae have a SMR about 84% of a general arthropod predicted value. Scorpions and ticks have very low SMRs, 24 and 12% respectively of the predicted arthropod value (Lighton and Fielden 1995, Lighton et al. 2001). Therefore, while huhu have a SMR below average, they cannot be categorised as having a very low SMR. Care needs to be taken when comparing published SMRs as much of the data lacks temporal resolution making measurement of inactive animals difficult (Lighton and Fielden 1995). Furthermore, Lighton's equation above was based on a limited number of taxonomic groups and the majority of studies used adult insects.

DGCs have been observed in a wide variety of insects, after initially being observed in motionless lepidopteran pupae (Levy and Schneiderman 1966, Lighton 1994, 1996). Paim and Beckel (1962) observed DGCs in cerambycid pre-pupae, pupae and a single moulting immobile larva. Whether the elevated carbon dioxide levels measured on some motionless huhu larvae were the result of DGCs, or some other process it is impossible to determine without the temporal resolution provided by flow-through respirometry.

The mean mass-independent \dot{M}_{O_2} (\pm SEM) of huhu larvae at 35°C measured manometrically and calculated from data reported in Chapter 3 was $3.13 \pm 0.14 \text{ nmol}\cdot\text{s}^{-1}$ ($n = 24$) and for a similar weight range measured using the automated system was $3.10 \pm 0.11 \text{ nmol}\cdot\text{s}^{-1}$ ($n = 14$). Measurements at 35°C were used for comparisons as huhu larvae are largely inactive at this temperature. Mean mass-independent \dot{M}_{O_2} (\pm SEM) at 20°C was 1.21 ± 0.073 ($n = 11$) and $1.32 \pm 0.032 \text{ nmol}\cdot\text{s}^{-1}$ ($n = 20$), respectively for manometric and gas analyser data. The manometric data at this temperature were limited, with fewer insects and observations compared to the automated system using gas analysers. There were no significant differences between either measurement method at both 35°C ($P = 0.90$) and 20°C ($P = 0.14$). Both measurement systems were capable of providing accurate and statistically the same measurements, however, the environmental conditions were different between them. In the manometric system, larvae were exposed to water-saturated air, whereas in the automated system, the air was initially dry but RH would have increased during the enclosure period as the larvae

lost water to the atmosphere prior to being purged with dry air during measurement. No carbon dioxide would have been present in the manometric system and the automated system would have cycled between zero and elevated levels as for humidity.

Conclusions

SMR was successfully measured for huhu larvae at 20, 30, 35 and 40°C according to published criteria (Lighton and Fielden 1995). SMR in huhu was lower than predicted values but within the range of non-flying adult coleopterans (Reinhold 1999, Lighton et al. 2001). Larval metabolism scaled with mass according to an exponent between 0.62 and 0.67 for this temperature range. As temperature increased the RQ also increased, suggesting a shift from primarily metabolising fat to include other substrates. Activity increased larval metabolism but RQ appeared to remain stable with increased activity at 20°C. Temperature sensitivity, as measured by mean Q_{10} and SMR, was similar whether measured by the automated gas analysis system or manometrically.

RESPIRATORY RESPONSE OF HUHU TO EXTREME STRESS

Introduction

Insects possess many behavioural responses that protect them from thermal injury, however, most species can only survive very high temperatures for short periods, which illustrates that mortality is a function of both temperature and time (Denlinger and Yocum 1998). There are many studies that document the mortality response of insects to heat (Hallman and Armstrong 1994, Denlinger and Yocum 1998, Tang et al. 2000). Dentener et al. (1999), for example, demonstrated that high mortalities in huhu larvae were associated with long times at 35°C but with increasingly short times at 40 and 45°C. Furthermore, there is comprehensive literature describing insect responses within their natural ecological temperature range (Lighton 1989, Lighton and Fielden 1995, Gillooly et al. 2001, Lighton et al. 2001, Addo-Bediako et al. 2002, Gillooly et al. 2002). Recently there has also been much interest in the induction of heat shock proteins (HSP) at sublethal temperatures, which confer thermo-tolerance at extreme temperatures (Chen et al. 1990, Denlinger et al. 1991, Yocum et al. 1991, Yocum and Denlinger 1992, Feder et al. 1997, Lester and Greenwood 1997). However, relatively few studies have sought to measure insect metabolic responses to extreme temperatures. One such study by Neven (1998) examined the effect of different temperature profiles (ramps) from ambient temperatures to maximums between 44 and 48°C on codling moth (*Cydia pomonella*) larvae. This study found a correlation between the time to reach a respiratory maximum and the time required to achieve a high level of mortality.

Hoback and Stanley (2001) defined hypoxia in terrestrial situations as any atmospheric oxygen concentration less than that in normal air (20.94%). From the literature it is clear that the term anoxia is typically used to describe a much lower oxygen concentration (but may be > 0%). Many insects spend various parts of their lifecycle in hypoxic /anoxic conditions as defined by their habitat, such as dung, soil, timber, within another organism, or due to some other environmental factor like submersion under water and ice, or exposure to high altitudes (Wegener 1993,

Becker et al. 1996, Hoback and Stanley 2001, Kolsch 2001, Kolsch et al. 2002). In this thesis I will be using anoxia to refer to exposure to pure nitrogen and following the definition of hypoxia of Hoback and Stanley (2001).

While mammals and insects are both typically highly aerobic, sustained hypoxia in mammalian tissues may result in irreversible damage or death, whereas many insects display a high degree of tolerance (Hochachka 1986, Wegener 1993). When insects are exposed to anoxic conditions there is typically a rapid loss of coordination resulting in paralysis, indicating anoxia has an impact on the central nervous system (Wegener 1993). In adult locusts, the heart stops, thereby preventing circulation of haemolymph. Weyel and Wegener (1996) discussed three potential metabolic strategies that insects can employ to maintain phosphorylation for the production of ATP when exposed to hypoxia; (1) Increase the anaerobic production of ATP, the inefficiency of which leads to a fast reduction in available carbohydrate substrate and the accumulation of acidic end products; (2) Decrease the metabolic rate; (3) Use alternate metabolic pathways to improve ATP yields. Typically, insects use strategy (2), often combined with (3) (Hochachka 1986, Wegener 1993). Microcalorimetric measurements on insects indicate that there is typically a rapid drop in metabolism upon exposure to severely hypoxic /anoxic conditions (Hochachka 1986, Weyel and Wegener 1996, Zhou et al. 2000, Kolsch 2001). In such a study on hawk moths, exposure to pure nitrogen for 15 h at 21°C resulted in heat production being reduced to 4% of the normoxic rate (Wegener 1993).

The effects of extreme temperatures and or controlled atmospheres on insects are commonly researched to develop treatments to disinfest products of insects (Carpenter and Potter 1994, Hallman and Armstrong 1994). Exposure to hypoxia or anoxia while a temperature stress is applied increases an insect's sensitivity to heat, leading to higher levels of mortality in disinfestation treatments (Whiting et al. 1991, Yocum and Denlinger 1994, Whiting and Hoy 1997). Dentener et al. (2001) found huhu larvae had a remarkable tolerance to pure nitrogen at 20°C but were killed in a comparatively short time in the same atmosphere at 40°C. However, exposure to anoxia prior to temperature stress induces thermotolerance in the locust flight system (Wu et al. 2002, Newman et al. 2003).

The primary objectives of this study were:

5. To determine the effect of time-temperature exposure to extreme temperature (up to 45°C) on larval gas exchange.
6. To determine if exposure to hypoxic atmospheres causes larvae to reduce their rate of gas exchange, consistent with a strategy of metabolic depression.
7. To determine if huhu larvae, while recovering from exposure to anoxia, display elevated metabolic activity, suggesting they have incurred an oxygen debt.

In this study, small and large huhu larvae were exposed to extreme constant temperature up to 45°C for extended periods while being subjected to respirometry to measure their metabolic response. Likewise, larvae were exposed to a temperature ramp to determine if they responded differently to a gradual increase in temperature compared with a thermal “shock” associated with being transferred from ambient conditions to an extreme temperature. Respiratory responses to a hypoxic atmosphere were measured to determine if larvae reduced their rate of gas exchange consistent with the strategy of metabolic depression. Respirometry was also conducted during recovery from anoxia to investigate if huhu follow a typical path of having to repay an oxygen debt. Respiratory responses to extreme stresses, such as those applied during commodity disinfestation, may be useful in the development of these treatments.

Materials and Methods

Insects

Huhu eggs were collected from felled trees in commercial pine plantations in Hawke's Bay, New Zealand. Insect collection, rearing and handling were as described in Chapters 2 and 3, with pre-experimental conditions as outlined in Chapter 4.

Respirometry

The automated insect respiration system described in Chapter 4 was used to measure oxygen consumption and carbon dioxide production of huhu larvae.

Heat treatment

Constant temperature

Huhu larvae were exposed to 35, 40, 42, 43, 44, and 45°C by immersing the respirometry chambers in a temperature-controlled water bath ($\pm 0.05^\circ\text{C}$ of the nominal temperature, RT200 resistance thermometer, Industrial Research, NZ). Respiration measurements (\dot{M}_{O_2} and \dot{M}_{CO_2}) of small and large larvae (see Chapter 4 for a description of larval size) were carried out for each temperature over a nominal period of five hours (Table 5.1).

Variable Temperature

Respirometry on large huhu larvae was carried out while the temperature was increased from 20 to 45°C in 270 min ($0.09^\circ\text{C}\cdot\text{min}^{-1}$) followed by a static temperature of 45°C for the remaining time. The enclosure period was 12 min and measurements were taken for each huhu every 13.5 min ($n = 21$ huhu larvae). The actual temperatures within two of the chambers were measured during the temperature ramps and used in the data analysis rather than the water bath temperature.

Table 5.1 Exposure temperature, size, mean mass, number and duration of huhu larvae used for respiration measurements.

Temperature (°C)	Size	Mean mass ± SEM (g)	n	Duration (min)
35	S	0.770 ± 0.030	14	302
	L	3.560 ± 0.074	21	292
40	S	0.641 ± 0.028	18	310
	L	3.593 ± 0.058	21	289
42	S	0.571 ± 0.021	14	310
	L	3.492 ± 0.057	21	302
43	S	0.581 ± 0.017	21	310
	L	3.305 ± 0.071	21	302
44	S	0.585 ± 0.016	14	310
	L	3.307 ± 0.060	21	302
45	S	0.588 ± 0.018	14	310
	L	3.516 ± 0.078	21	302

S = Small, L = Large

Hypoxic atmospheres

Huhu larvae were exposed to either pure nitrogen (gas code 152, BOC Gases NZ Ltd) for 55.5 min plus 5 minutes purging with air followed by 4 hours of respirometry with normal baseline air, or 1.78% oxygen (balance nitrogen, 163GS, Specialty Gases BOC) for 5 hours while measuring carbon dioxide production. Equipment limitations prevented oxygen measurements at such low levels without substantial hardware and software modifications. Measurements on huhu at 1.8% oxygen were made at 20 and 40°C. Q_{10} was calculated according to the formula,

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{\frac{10}{T_2 - T_1}},$$

where R_2 and R_1 are the metabolic rates at temperatures T_2 and T_1 and Q_{10} is the change in rate caused by a 10°C increase in temperature (Schmidt-Neilson 1979). Recovery after exposure to nitrogen was measured at 40°C only, because at 20°C huhu began to move immediately after returning to normoxic conditions, thereby compromising the measurements.

Statistics

Multiple measurements were taken from the same huhu larva over a period of time, in addition to replication provided by measuring multiple larvae. This type of data is referred to as longitudinal data, and requires a statistical approach that considers these repeated measures. Repeated measurements on the same subject tend to be correlated and therefore, independence cannot be assumed (Potvin et al. 1990). Consequently the error covariance structure of the data needs to be modelled to prevent flawed statistical inferences being drawn from the analysis.

The repeated measures module of Genstat (Genstat V.7.2, VSN International Ltd, UK) using the residual maximum likelihood (REML) function on response data (Log_{10} transformed when necessary) was used to determine significance and estimate means. An appropriate covariance model was selected using deviance to determine the best fit. The Wald statistic was used to test the significance of the fixed effects terms as they were added to the model. The Wald statistic is the ratio of the square of the estimated parameter value to the curvature of the likelihood function. It has an asymptotic χ^2 distribution with degrees of freedom equal to that of the parameter. Different time points for large and small huhu larvae prevented analysis as a single data set. Therefore after separate analyses, oxygen and carbon dioxide values were predicted at 3 common time points using REML with a fixed model that incorporated temperature and time using a cubic polynomial. An appropriate SED was calculated from the predicted errors and means compared using LSD ($P < 0.05$). Response surface graphs were generated with Origin™ (Version 7.5, Microcal Software Inc., MA, USA) and used the Renka-Cline method to develop the 3-dimensional plotting matrices.

Response data from the variable temperature ramps were analysed using a repeated measures approach in Genstat with the fixed model incorporating either a quadratic or cubic polynomial. The first derivative of the polynomials were set to zero and solved for time (x) to identify the peak, which enabled a REML prediction of the response value and the calculation of temperature for this time. To determine \dot{M}_{O_2} at specific temperatures for plotting, a time-temperature model was fitted followed by REML predictions at the temperature of interest.

Results

Respirometry at elevated temperatures

Constant temperature

REML analysis using a repeated measures approach indicated that temperature, elapsed time and the interaction of time and temperature all had a significant effect on \dot{M}_{O_2} , \dot{M}_{CO_2} and RQ for both small and large size classes of huhu larvae (Table 5.2). Mean huhu \dot{M}_{O_2} of small larvae increased uniformly with temperature from 35 to 42°C and varied little with elapsed time (Figure 5.1A). However, at temperatures above 42°C there were significant changes in slope with elapsed time, which were greatest at 45°C where \dot{M}_{O_2} values declined to 6.0% of initial measured values. A similar pattern was seen with \dot{M}_{CO_2} of small larvae, except huhu exposed to 42 to 45°C appeared to reach a maximum limit of carbon dioxide production (Figure 5.1B). Large huhu larvae had similar \dot{M}_{O_2} and \dot{M}_{CO_2} responses to temperature with elapsed time as small larvae (Figures 5.2A and B), although the slope changed for large larvae at 42°C compared with 43°C for small larvae, for both \dot{M}_{O_2} and \dot{M}_{CO_2} .

The varying patterns of oxygen consumption with time and temperature for small and large huhu, are shown as 3-dimensional surface plots in Figures 5.3A and B, respectively. The overall trends of high larval \dot{M}_{O_2} associated with high temperature and short exposure times, and lowest \dot{M}_{O_2} associated with high temperature and long exposure times were evident for both size classes of huhu. However, small huhu had a more pronounced critical time-temperature peaked response, which equated to the maximum \dot{M}_{O_2} for any given time. Large huhu had a visible peak at shorter times, but as exposure time increased, the response formed a plateau which then fell with increasing temperature. After 300 min exposure, the critical temperature, as indicated by a decrease in \dot{M}_{O_2} , was 42 to 43°C, and 43 to 44°C, respectively, for small and large huhu. This difference in response suggests small larvae were less tolerant of extreme temperatures after this exposure time.

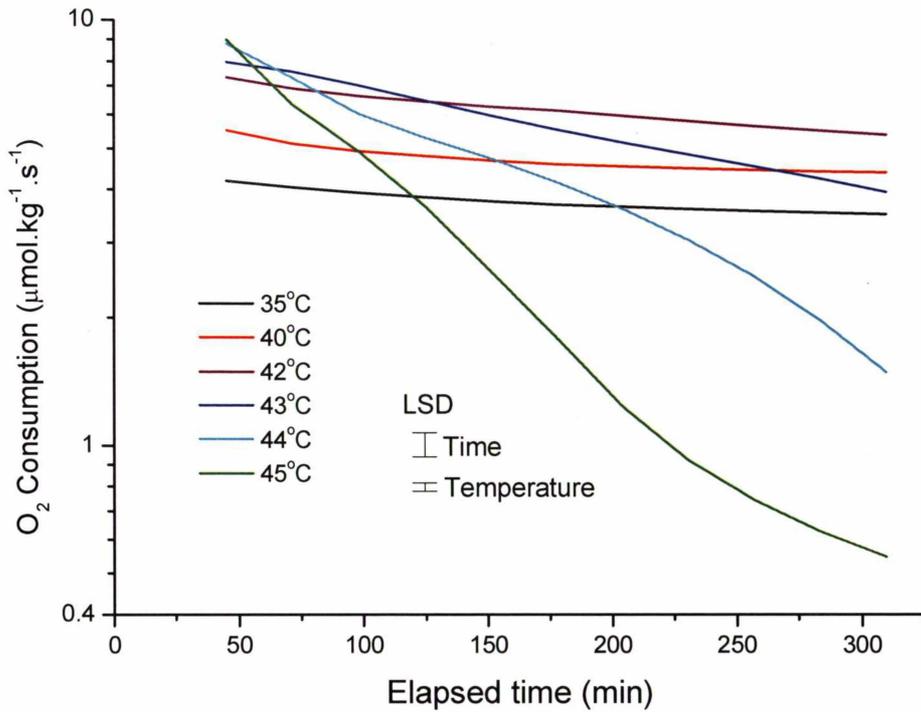
Table 5.2 Table of effects for small and large huhu larvae (analysed separately) exposed to 35 to 45°C, all factors and interactions were significant, $\chi^2 P < 0.001$.

Size	Parameter	Fixed factor	Wald Statistic	df	Wald Statistic/df
Small	\dot{M}_{O_2}	Time	5792	10	579
		Temperature	666	5	133
		Time. Temperature	6351	50	127
	\dot{M}_{CO_2}	Time	6997	10	700
		Temperature	765	5	153
		Time. Temperature	6375	50	128
	RQ	Time	354	10	35
		Temperature	191	5	38
		Time. Temperature	421	50	8
Large	\dot{M}_{O_2}	Time	32894	20	1645
		Temperature	1094	5	219
		Time. Temperature	25440	99	257
	\dot{M}_{CO_2}	Time	44692	21	2128
		Temperature	864	5	173
		Time. Temperature	17676	99	179
	RQ	Time	6014	20	301
		Temperature	150	5	30
		Time. Temperature	2207	99	22

Mean \dot{M}_{O_2} and \dot{M}_{CO_2} of small and large huhu at different temperatures are compared for three elapsed times in Tables 5.3 and 5.4. At almost all temperature-time combinations small larvae have significantly higher \dot{M}_{O_2} and \dot{M}_{CO_2} values and were only statistically equivalent at high temperatures with long exposure times.

Figure 5.1 Response of small huhu larvae at constant temperatures; (A) mean oxygen consumption; (B) mean carbon dioxide production. LSDs, $P = 0.05$, are shown for the same level of time and temperature (see Table 5.1 for n values).

A.



B.

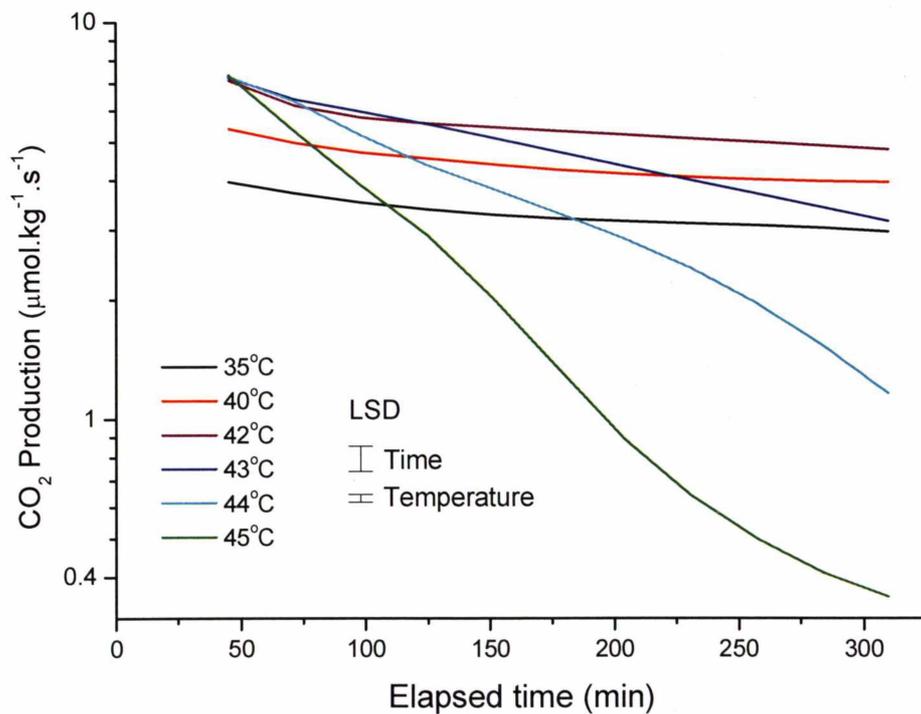
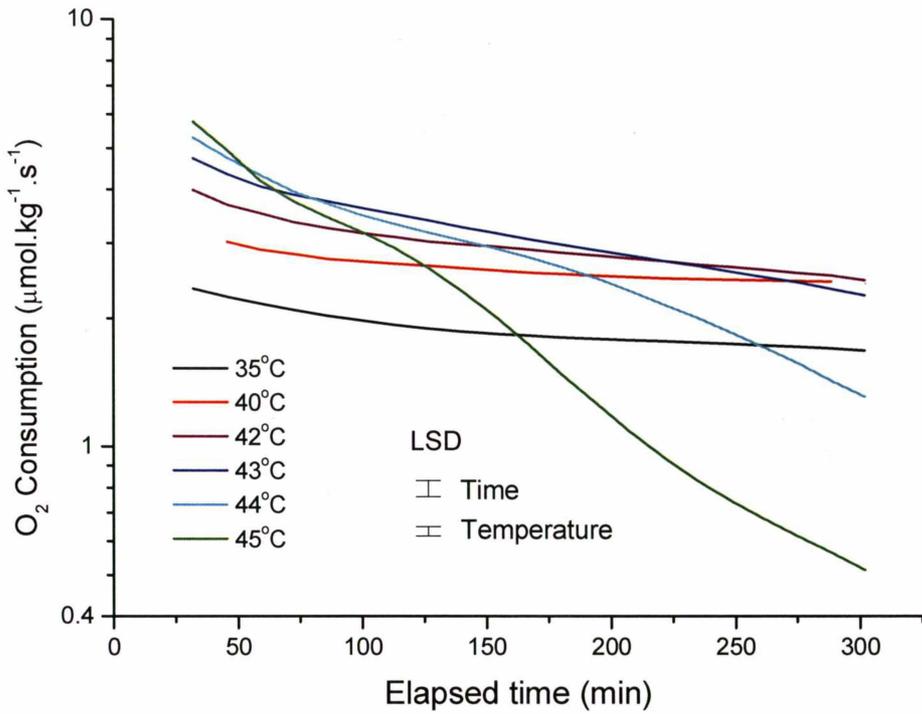


Figure 5.2 Response of large huhu larvae at constant temperatures; (A) mean oxygen consumption; (B) mean carbon dioxide production. LSDs, $P = 0.05$, are shown for the same level of time and temperature (see Table 5.1 for n values).

A.



B.

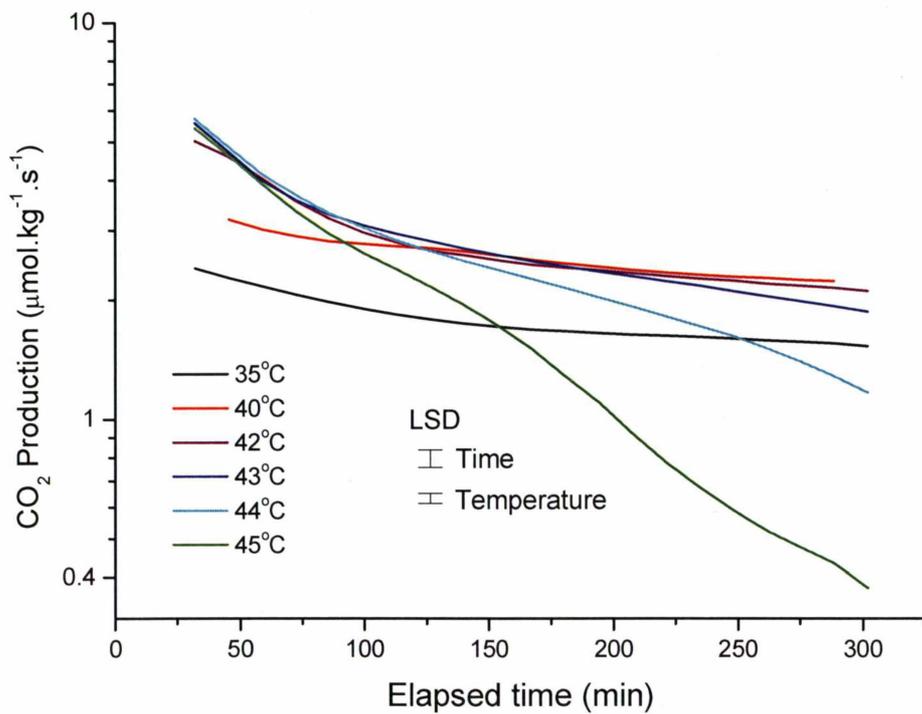
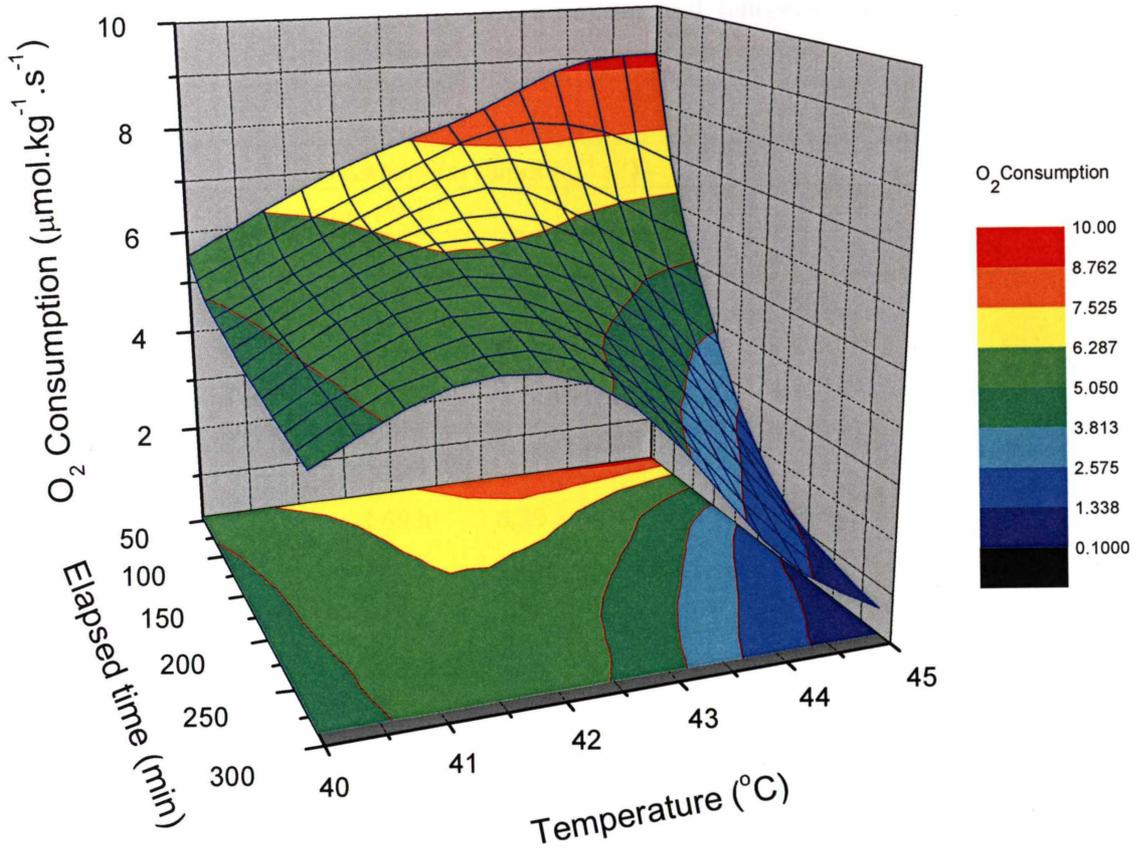


Figure 5.3 Mean oxygen consumption of huhu by time and temperature displayed as a 3-dimensional response surface; (A) small larvae; (B) large larvae.

A.



B.

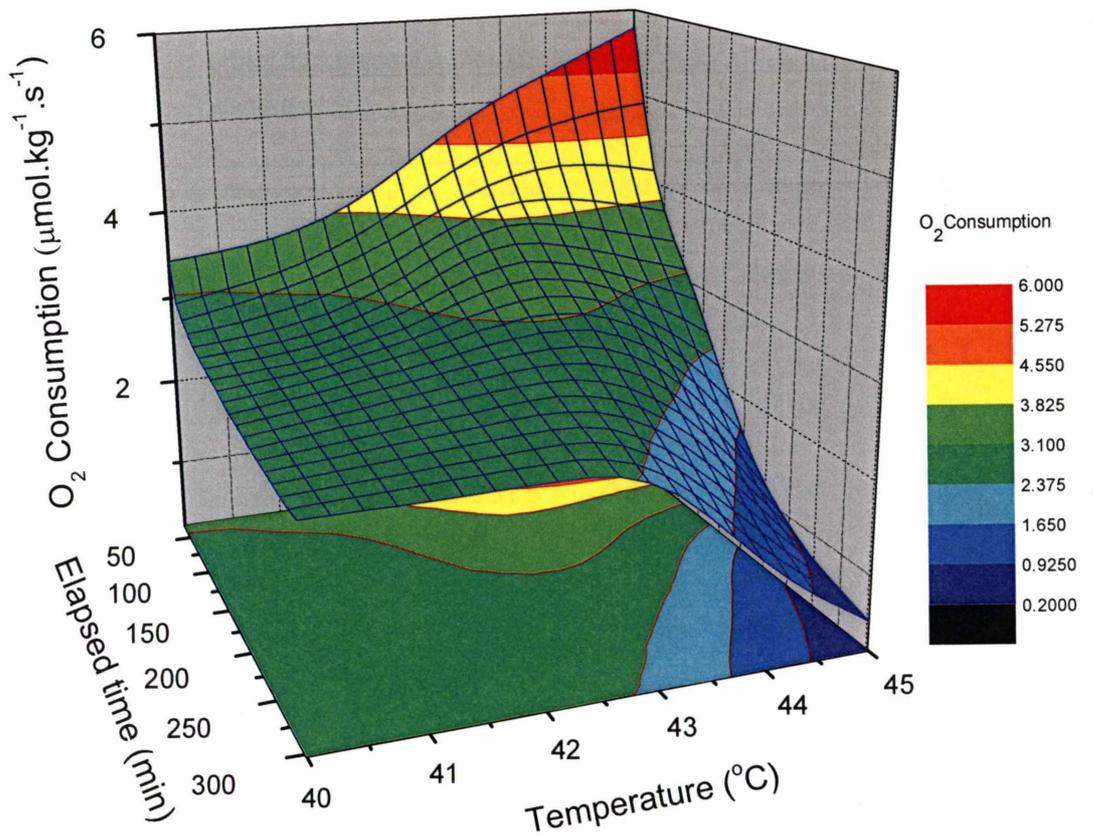


Table 5.3 & 5.4 Mean predicted respective oxygen consumption and carbon dioxide production of small (S) and large (L) huhu larva at different temperature and time exposures. Means not followed by the same letter are significantly different ($P < 0.05$) for the same level of time (rows, letters beside) and temperature (columns, letters below).

		Oxygen consumption ($\mu\text{mol.kg}^{-1}.\text{s}^{-1}$)					
Elapsed time (min)	Size	Temperature ($^{\circ}\text{C}$)					
		35	40	42	43	44	45
45	S	4.20 cd a	5.52 b a	7.37 a a	8.01 a a	8.84 a a	9.02 a a
	L	2.25 f b	3.12 e c	3.73 de c	4.39 cd c	4.75 bc b	4.94 bc b
150	S	3.79 c a	4.69 b ab	6.29 a ab	6.10 a b	4.75 b b	2.51 c c
	L	1.85 f c	2.58 e d	2.94 de d	3.20 cd d	2.92 de c	1.99 f d
300	S	3.54 c a	4.45 ab b	5.50 a b	4.08 bc c	1.67 e d	0.56 g e
	L	1.69 e c	2.39 d d	2.46 d d	2.28 d e	1.30 f e	0.51 g e

		Carbon dioxide production ($\mu\text{mol.kg}^{-1}.\text{s}^{-1}$)					
Elapsed time (min)	Size	Temperature ($^{\circ}\text{C}$)					
		35	40	42	43	44	45
45	S	3.97 de a	5.39 c a	7.14 ab a	7.24 ab a	7.30 ab a	7.36 a a
	L	2.28 f c	3.36 e c	4.49 cd b	4.69 cd b	4.85 c b	4.55 cd b
150	S	3.28 d a	4.35 abc b	5.31 a b	5.06 ab b	3.89 cd c	1.93 fg c
	L	1.72 g d	2.53 e d	2.51 e c	2.56 e d	2.37 ef d	1.67 g c
300	S	2.98 c b	3.96 ab b	4.84 a b	3.25 ba c	1.29 ef e	0.37 g d
	L	1.53 e d	2.12 d d	2.08 d c	1.83 d e	1.17 f e	0.37 fg d

The mean larval RQ declined slightly over time at most temperatures (Figures 5.4A and B). Large larvae exposed to 42, 43 and 44°C initially had much higher RQs, greater than 1.0, prior to stabilising between 0.8 and 0.9. Small larvae exposed to 42°C and large larvae exposed to 44°C were both atypical, having significant increases in RQ after an initial decrease. The RQ at 45°C had the lowest value after 300 min for both large and small larvae.

Mean weight loss of large huhu larvae was $3.39 \pm 0.21\%$ (\pm SEM; $n = 14$) after 5 h of respirometry at 45°C with the system supplied with dry air. Weight loss in huhu during respirometry was assumed be equivalent to water loss.

Variable Temperature

Huhu larval respiration rates increased in response to an increasing temperature ramp of 0.09°C per min (Figure 5.5). However, larval activity caused \dot{M}_{O_2} and \dot{M}_{CO_2} to vary at temperatures less than about 35°C, which is illustrated by representative data, shown in Figures 5.5A and B, respectively, of an individual huhu that remained mostly immobile and one that moved vigorously. The temperature ramp increased the temperature from 20 to 45°C at a constant rate for 270 minutes followed by a static temperature of 45°C for the remainder of the time. The larval response to this linear increase in temperature appeared to be curved rather than linear based upon a small number of larvae that displayed minimal movement. Larval \dot{M}_{O_2} and \dot{M}_{CO_2} peaked in response to this temperature ramp, however, to analyse this data further, measurements after 194 min, which equates to 38°C, were selected after movement had ceased. REML analysis using a repeated measures approach indicated time and time² were both significant ($P < 0.001$) as they were sequentially added to the quadratic model for both \dot{M}_{O_2} and \dot{M}_{CO_2} . Larval \dot{M}_{CO_2} peaked earlier than \dot{M}_{O_2} and hence at a lower temperature, while \dot{M}_{O_2} peaked about the same time that the ramp ended and the constant temperature of 45°C was reached (Table 5.5).

Because RQ appeared to be unaffected by movement (see Chapter 4) a cubic model was fitted for all the data. The predicted RQ at time = 30 min was 0.82 ± 0.026 (23°C) which gradually increased to a maximum of 1.0 as shown in Table 5.5. All the cubic polynomial terms were significant as they were sequentially added to the fixed model ($P < 0.001$).

Table 5.5 Response of large huhu larvae to a heating ramp of 0.09°C per min. Polynomial coefficients describe the quadratic model of the form $A+Bt+Ct^2$ for oxygen consumption and carbon dioxide production, with a cubic used for RQ with the additional term Dt^3 , where t = time ($n = 21$ huhu larvae).

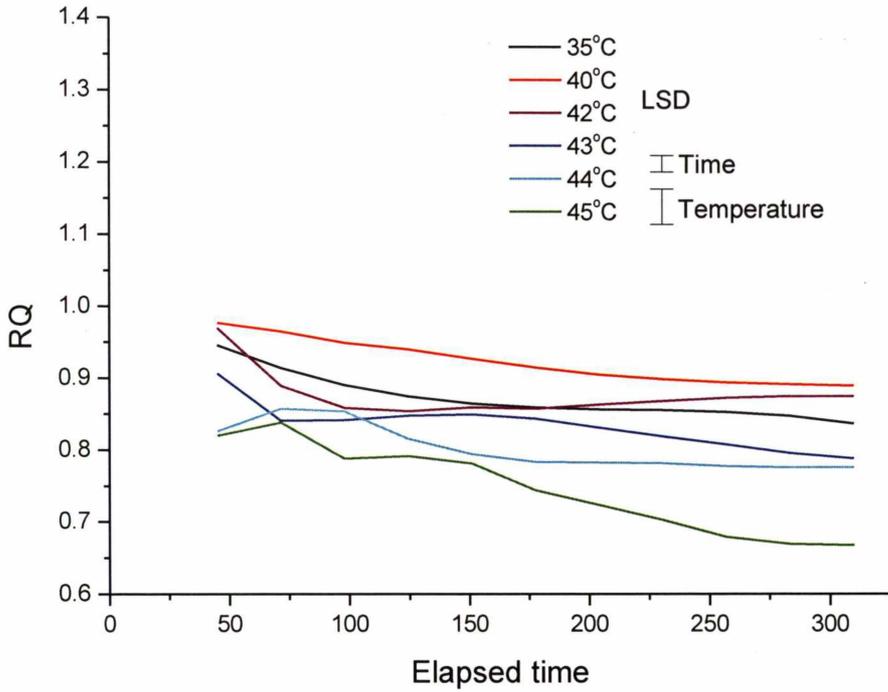
	\dot{M}_{O_2}	\dot{M}_{CO_2}	RQ ¹
Polynomial coefficients			
A	-14.3 ± 1.68	-21.0 ± 1.81	0.855 ± 0.050
B	0.141 ± 0.014	0.199 ± 0.015	-0.125 ± 0.070
C	-2.64E-04 ± 2.67E-05	-3.91E-04 ± 2.88E-05	0.103 ± 0.028
D			-0.0153 ± 3.19E-03
Time to peak (min)	266	255	228
Peak value ($\mu\text{mol.kg}^{-1}.\text{s}^{-1}$)	4.40 ± 0.083	4.47 ± 0.091	1.035 ± 0.018 ²
Temperature at peak (°C)	44.8	43.7	41.3

¹ Coefficients based on time in h

² Dimensionless units

Figure 5.4 Mean RQ of huhu at constant temperatures; (A) small larvae; (B) large larvae. LSDs, $P = 0.05$, are shown for the same level of time and temperature (see Table 5.1 for n values).

A.



B.

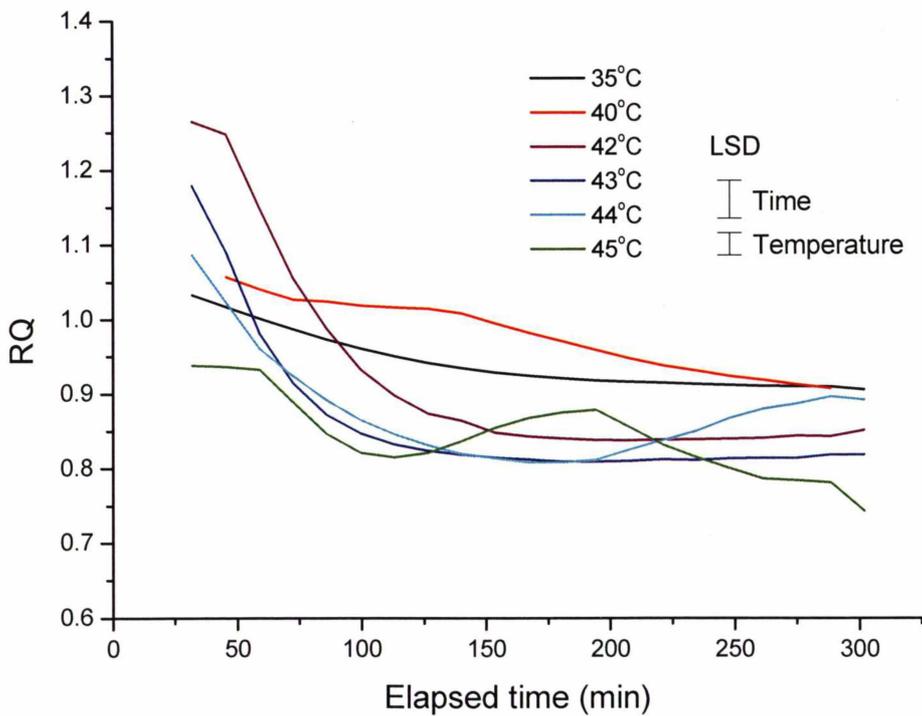
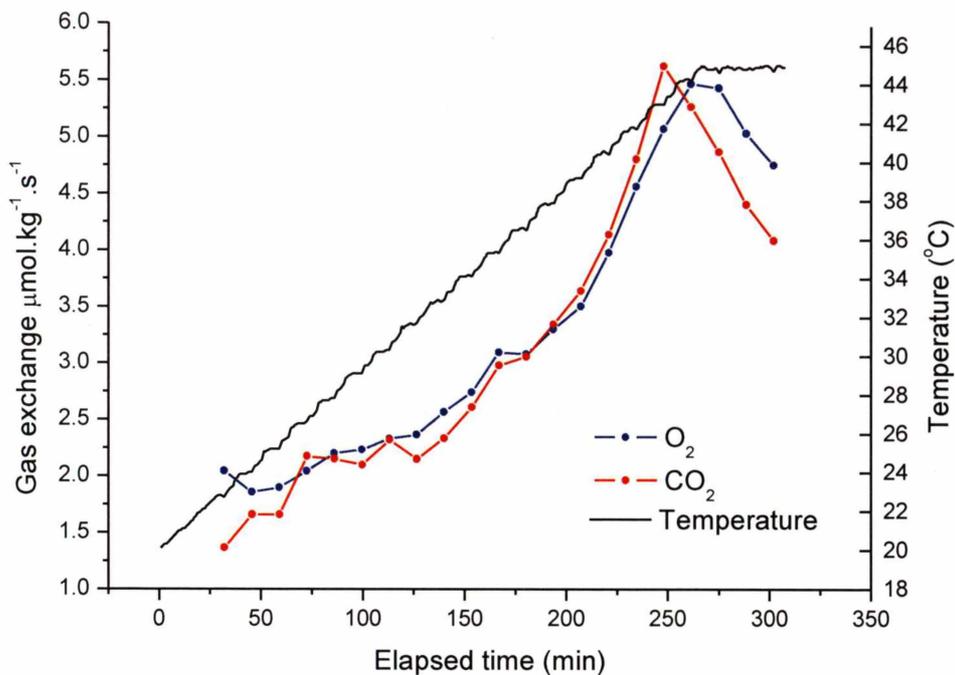
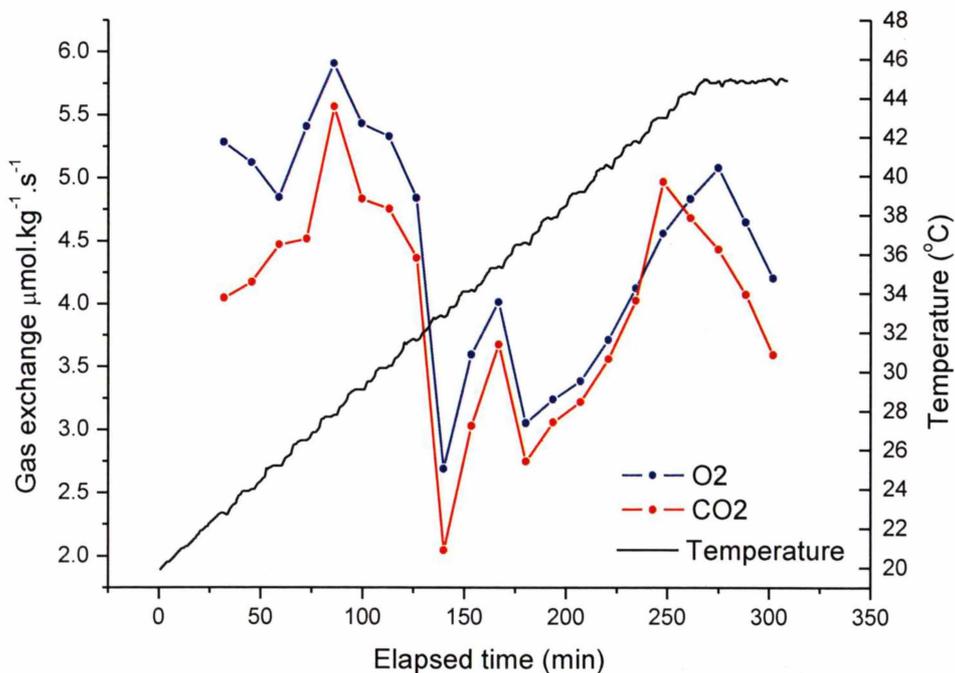


Figure 5.5 Oxygen consumption and carbon dioxide production of large huhu exposed to increasing temperature; (A) a larva that remained mostly inactive; (B) an active larva.

A.

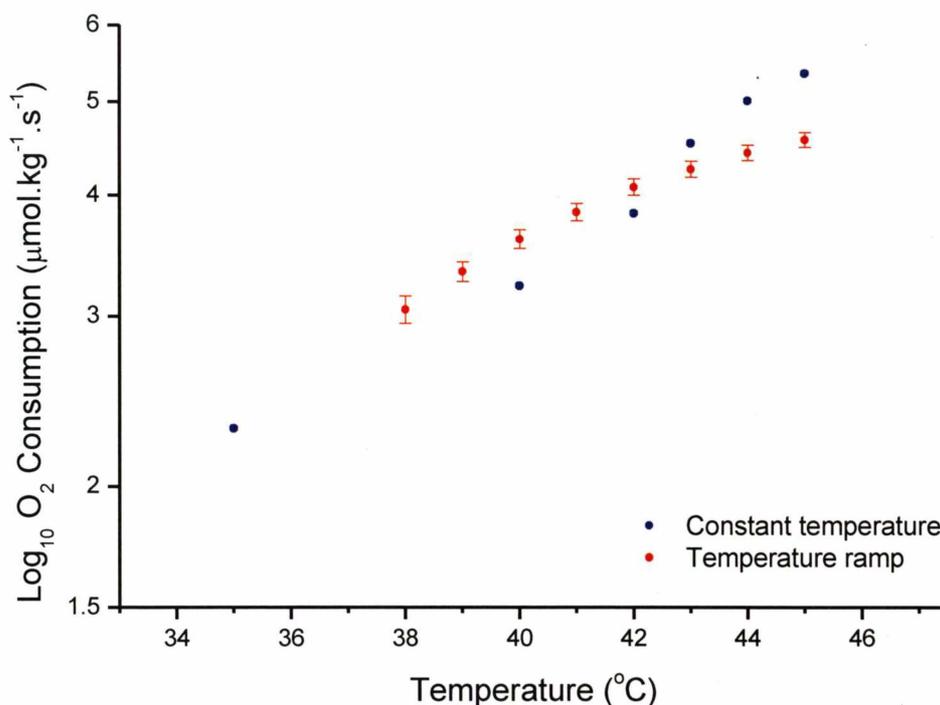


B.



\dot{M}_{O_2} increased with temperature when large huhu larvae were exposed to a temperature ramp at times greater than 194 min with a response similar to constant temperature (Figure 5.6). Repeated measures REML analysis using a fixed model, which included time, temperature and time-temperature terms, indicated all terms had a significant effect on \dot{M}_{O_2} ($P < 0.05$).

Figure 5.6 Oxygen consumption of large huhu larvae exposed to constant temperatures (time = 32 min) and a temperature ramp of 0.09°C per min. Error bars denote SEM of the predicted values.

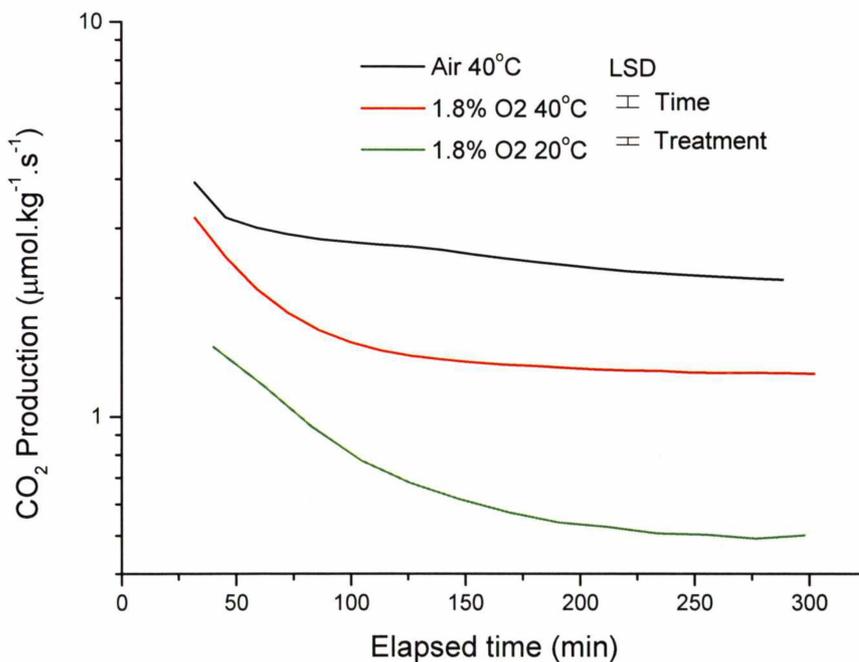


Hypoxic atmospheres

Reducing the oxygen concentration to 1.8% significantly decreased the carbon dioxide production of large huhu larvae at 40°C (Figure 5.7). Initially the reduction was quite small but \dot{M}_{CO_2} continued to decrease until stabilising after about 125 min. Exposure to 1.8% oxygen at 20°C showed a similar pattern but had a more gradual decrease and didn't stabilise until about 250 min. Comparing stabilised values after 275 min for all treatments with stable measurements (SMR, Chapter 4) at 20°C in air demonstrated that atmosphere, temperature and their interaction all had a significant effect on \dot{M}_{CO_2} (Table 5.6). Exposure of huhu to 1.8% oxygen at 20°C resulted in a stabilised mean \dot{M}_{CO_2} that was 79% of the value of huhu in air at 20°C, however, this was not significantly different. However, exposure to this

reduced oxygen atmosphere prevented all larval movement, after an initial adjustment period which contrasts with often excessive movement at 20°C in air. Exposure to 40°C and 1.8% oxygen led to a 58% reduction in larval \dot{M}_{CO_2} compared to standard air at this temperature. The Q_{10} for the temperature increase from 20 to 40°C at 1.8% oxygen was 1.62 compared to 1.90 for air reported in Chapter 4.

Figure 5.7 Mean carbon dioxide production of large huhu larvae exposed to 1.8% oxygen atmospheres at 20 and 40°C (n = 14 huhu). The response to normal air at 40°C is shown for comparison (n = 21 huhu). LSDs, $P = 0.05$, are shown for the same level of time and treatment.

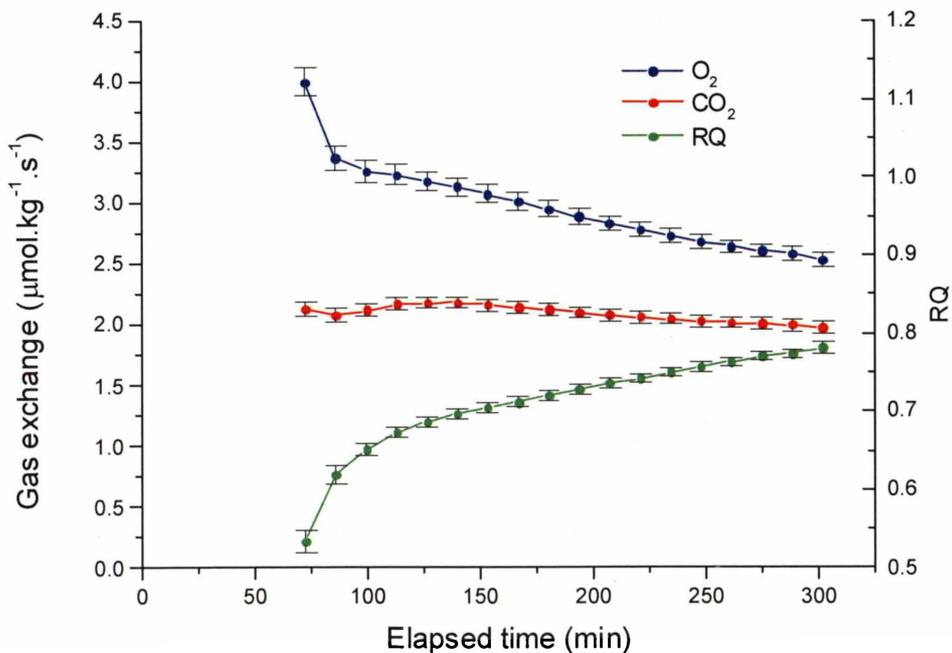


Metabolic measurements during recovery of huhu larvae after exposure to nitrogen at 40°C showed elevated oxygen consumption which, after an initial drop, fell at a constant rate, and stable carbon dioxide production (Figure 5.8). Consequently, the RQ initially increased rapidly followed by a gradual increase. After 300 min exposure to 40°C (including 55.5 min of nitrogen), metabolism had not returned to predicted values for 40°C air exposure.

Table 5.6 Mean carbon dioxide production of large huhu larvae exposed to standard air and 1.8% oxygen at 20 and 40°C after 275 min. Measurements on huhu in air at 20°C were an exception where a mean was calculated per insect from immobile huhu and reported in Chapter 4. All treatments were significantly different ($P < 0.05$) except between atmospheres at 20°C.

\dot{M}_{CO_2} ($\mu\text{mol.kg}^{-1}.\text{s}^{-1}$)		
Atmosphere	20°C	40°C
1.8% O ₂	0.498	1.303
20.7 – 21.2% O ₂	0.628	2.257
LSD 0.160, $P = 0.05$		
F ratio		
Atmosphere	114	< 0.001
Temperature	527	< 0.001
Interaction	53	< 0.001

Figure 5.8 Mean oxygen consumption, carbon dioxide production and RQ of large huhu larvae at 40°C, after exposure to pure nitrogen for 1 h (error bars denote SEM, n = 14 huhu).



Discussion

Elevated temperatures

Typically, insect metabolic processes are dependent on environmental temperature because they are exothermic poikilotherms. The significant effects of time and temperature on huhu metabolism at elevated temperatures in this study were expected and consistent with how poikilotherms respond to heat (Schmidt-Neilson 1979, Neven 2000, Gillooly et al. 2001, Gillooly et al. 2002). The interaction of time and temperature was also significant indicating the effect of temperature on metabolism differed with time and vice versa.

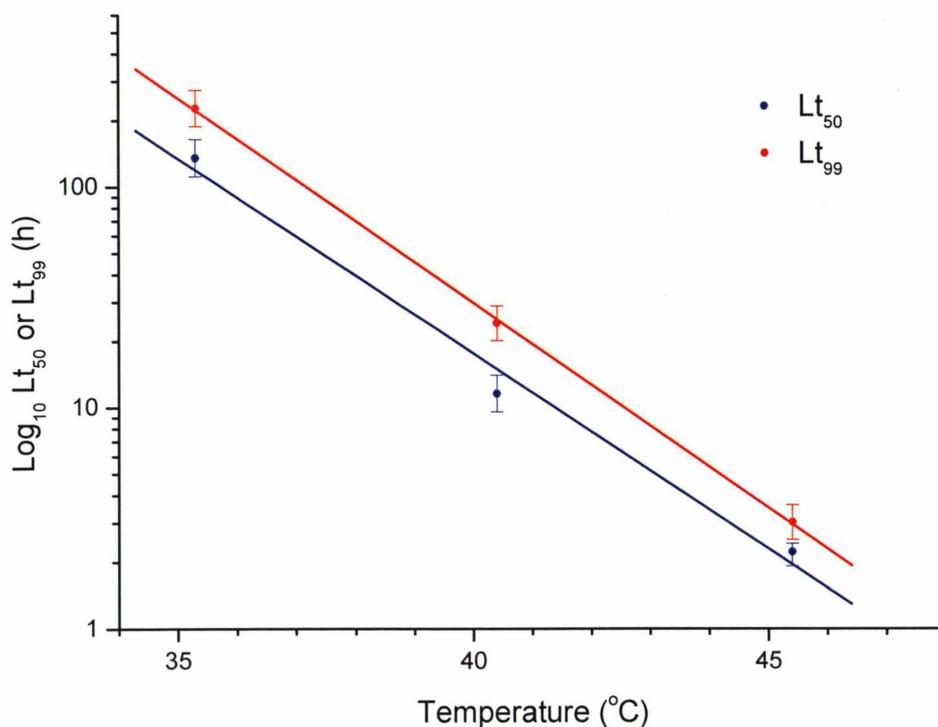
At temperatures less than about 42°C, huhu larval metabolic rate, as indicated by oxygen consumption, increased with increasing temperature, which is consistent with other insect studies (Schmidt-Neilson 1979, Neven 1998, Lighton et al. 2001). However, there was a critical temperature for huhu as indicated by a change in slope when \dot{M}_{O_2} was plotted on elapsed time, after which, metabolic rate decreased at a greater rate with time. Increases of metabolic dependent processes to critical thermal limits followed by a decrease are described for many insect species (Wigglesworth 1972, Schmidt-Neilson 1979, Denlinger and Yocum 1998, Neven 2000). However, in this study it was not possible to determine if this decrease in respiration was caused by depression of the metabolic rate to conserve fuel in response to increasing temperature, or the direct effect of temperature causing damage to the insect, or a combination of both processes.

Critical thermal limits are also seen in insect mortality studies at high temperatures, where survival curves (survival plotted against time) typically have two phases, a broad shoulder with low mortality followed by high mortality (Denlinger and Yocum 1998). Huhu larval mortality data described by Dentener et al. (1999) displayed the same type of response, where lethal times to 99% mortality (L_{t99}) and L_{t50s} associated with low temperatures were very long, but reduced to very short periods for a small increase at high temperatures (Figure 5.9). Huhu larvae had an L_{t99} of 3 h (2.5-3.6, 95% CI) at 45°C (Dentener et al. 1999), which is consistent with the respiratory changes with time between 42 and 45°C described above. However, these mortality data for huhu also suggest the larvae may have been dead during respirometry at 45°C, especially as other studies have found that organismic and tissue gas exchange can still occur after death (Denlinger and Yocum 1998, Neven 1998).

Determining mortality of insects can be quite difficult. For huhu larvae, Dentener et al. (1999) defined death as failure to move when prodded, 3-4 d after heat treatment. Flesh flies exposed to a 45°C heat treatment can continue to develop normally but ultimately fail to escape from the puparium and die, leading to the description of the “living dead”, with mortality defined as those flesh flies that failed to emerge (Chen et al. 1990, Denlinger and Yocum 1998).

In this study, huhu post-treatment larval mortality was not assessed for three main reasons. Firstly, the same huhu larvae had haemolymph samples withdrawn immediately after removal from the respirometry chambers, which could have compromised larval survival, as they would often continue to bleed excessively. Secondly, the sample sizes were too small to provide meaningful mortality data. Finally, even if mortality was determined after the treatment and larvae were found to be dead, their status during respirometry would still be unknown. However, if larvae were dead after long exposures to high temperatures, the respirometry data would still be useful for the development of heat-based quarantine treatments, as the measurements would pertain to the process of dying.

Figure 5.9 Lt_{50} and Lt_{99} response of huhu larvae > 100 mg at three temperatures. Data plotted from Dentener et al. (1999), errors denote 95% CIs.



In contrast to oxygen consumption, carbon dioxide production appeared to reach a maximum level with increasing temperature, especially during initial exposure for both small and large, but was also evident at later times for large larvae. These data suggest that elimination of carbon dioxide at elevated temperatures may be a physiologically limiting factor. However, any contribution this may have to sublethal stress or mortality would be speculative.

While small and large huhu larvae have the same overall pattern of metabolic response to extreme temperatures, small larvae typically had higher mass specific metabolic rates. This was consistent with data at lower temperatures (Chapters 3 and 4) and other studies on animals (Schmidt-Neilson 1979, 1984); although interestingly at high temperatures and long exposures, metabolic rates were statistically the same. Small larvae appeared to be more thermo-tolerant compared to large, as indicated by changes in slope, at short exposure times, whereas at long exposures, this was reversed. Overall, small larvae appear to be less thermo-tolerant than large, especially when the percentage change in metabolism is considered with increasing time-temperature exposure.

Initial RQ values at 42-44°C for large larvae were much higher prior to stabilising, and suggest these larvae were slower at adapting to what Neven (2000) described as a thermal challenge associated with a step-function transfer; that is, a transfer from a lower static temperature to a higher one. At temperatures greater than 40°C, the general trend for both small and large huhu larvae was for the RQ to decrease, which contrasts with the trend of increasing RQ for 20-40°C described in Chapter 4. This provides further evidence that the response of huhu to thermal stress below 40°C was very different to above this temperature. At 45°C, the RQ of small larvae after initially stabilising showed a gradual decline consistent with apparent greater temperature sensitivity compared with large larvae, while in large larvae at 45°C, the RQ fell, stabilised, rose, peaked and then fell for unknown reasons.

At high temperatures it is difficult to separate the physiological effects of temperature and desiccation, unless relative humidity is close to saturation (Denlinger and Yocum 1998). Therefore, since this study used dry air for respirometry at high temperatures, interpreting the results in terms of temperature only, is a potential criticism. However, while huhu larvae were initially exposed to dry air from the incurrent air, within the relatively small chamber humidity would have increased during the enclosure period. Also, water loss of huhu, as indicated

by weight loss of large larvae exposed to 45°C, was minimal, suggesting they may be one of the many species well adapted to low humidity environments (Wigglesworth 1972). Many insects can tolerate losing up to 20-30% of their water (total water composition 60-70%) during short exposures to desiccating conditions (Hadley 1994). If desiccation were having a major impact on huhu respiration at high temperatures, the response of small larvae, by virtue of their surface area to volume ratio, would be very different to large larvae. But in this study the pattern of responses of small and large larvae were similar. Furthermore, the mean \dot{M}_{O_2} of larvae exposed to 45°C measured using manometric methods (see Chapter 3) in a water-saturated atmosphere after 195 min was similar to the mean using automated respirometry with dry air. The respective means (\pm SEM) were 1.76 ± 0.17 (n = 12 huhu) and $1.42 \mu\text{mol.kg}^{-1}.\text{s}^{-1}$ (0.153 ± 0.016 , Log_{10} transformed REML prediction n = 14 huhu).

Due to the technical constraints of using water-saturated air for respirometry, most open-system respirometry on insects utilizes dry air (Lighton 1991a). Studies of water relationships in ticks have demonstrated that low humidity can affect the frequency of respiratory cycles and lead to increases in metabolic rate (Fielden and Lighton 1996). In contrast, while the desert harvester ant increased its metabolic rate in response to dry air, this was demonstrated to be caused by increased activity as part of an escape response (Lighton and Bartholomew 1988). Most water loss in insects, especially at high temperatures, takes place through the cuticle rather than via the spiracular system. Therefore the quantity and type of cuticular hydrocarbons are very important (Ahearn 1970b, Yoder and Denlinger 1991, Denlinger and Yocum 1998, Salin et al. 1999).

Huhu larval \dot{M}_{O_2} and \dot{M}_{CO_2} increased with increasing temperature when exposed to a temperature ramp, leading to a peaked response prior to falling, consistent with similar studies on codling moth larvae (Neven 1998). Mean maximum \dot{M}_{O_2} corresponded to the transition of the temperature ramp to the target constant temperature of 45°C. This suggests this peak was probably artificially produced by the temperature ramp. However, \dot{M}_{CO_2} peaked before the target temperature was reached and was followed by an additional reading prior to 45°C. Therefore, this \dot{M}_{CO_2} peak represented the stress response of larvae to this particular heating ramp. Larval movement at temperatures less than about 35°C prevented analysis of the entire time period and hence calculation of a Q_{10} response. The \dot{M}_{O_2} larval response to this particular temperature ramp was similar to the initial response to

constant temperatures. In contrast, Neven (1998) found the upper respiratory limit of codling moth for variable temperature profiles was more than twice what was predicted from constant temperature regression. However, the temperature range for constant temperature respirometry was 10-30°C, which was used to predict responses for comparisons with temperature ramps with target temperatures of 44-48°C. The RQ for huhu exposed to a temperature ramp increased for each larva as the temperature increased, reaching a peak at 41°C. This pattern was similar to that described in Chapter 4 for different larvae exposed to different constant temperatures and indicates a change in metabolic substrate in response to heat stress. Also, the decline in RQ at temperatures greater than 40°C was consistent with constant temperature exposures described above.

Huhu response data to time-temperature exposure were analysed using a repeated measures approach, because standard analysis of variance and regression requires independent measurements. Potvin and Lechowicz (1990) highlighted the need for different approaches incorporating repeated measures to be used by physiological ecologists in their data analysis. Yet a review of papers published between 1991 and 2002 of the photosynthetic responses to light and carbon dioxide in 16 ecological journals found 56 papers that used a variety of analyses but only one incorporated repeated measures (Peek et al. 2002). The situation appears to be similar in insect physiology. In contrast, medical and veterinary science studies typically use repeated measures models in their analyses, for examples and reviews see Potvin et al. (1990), Littell et al. (1998), Peek et al. (2002) and Ketelaere et al (2003).

Hypoxic atmospheres

Reducing the oxygen concentration from normoxic to the hypoxic level of 1.8% resulted in reduced metabolic rates in huhu larvae as measured by carbon dioxide production at 20 and 40°C. However, this reduction was not significant at 20°C. These results are consistent with respirometry studies on stored-product pests which also found reduced gas exchange under hypoxic conditions (Emekci et al. 2002, 2004). Metabolic depression, sometimes inaccurately described as metabolic arrest, is used by many invertebrates as a strategy for surviving hypoxia and anoxia (Hochachka 1986, Wegener and Moratzky 1995, Hoback and Stanley 2001, Kolsch et al. 2002). Microcalorimetric studies on locusts have demonstrated that heat flows are reduced to 5% of normoxic rates under strict anoxia and that heat flow rates were correlated to the degree of hypoxia (Wegener and Moratzky 1995). While

hypoxia clearly resulted in reduced gas exchange in huhu larvae, respirometry, unlike calorimetry, does not measure any anaerobic component of metabolism that can occur during hypoxia. Larvae exposed to hypoxic conditions at 20°C ceased moving, which can be interpreted as an effective response to conserve metabolic reserves under adverse conditions. Yet clearly at 1.8% oxygen, gas exchange could still occur, in stark contrast to most vertebrates (Hochachka 1986, Wegener 1993). Decaying wet beech logs with internal atmospheres as low as 1% oxygen do not interfere with normal movement and feeding behaviour in *Orthosoma brunneum* cerambycid larvae (Paim and Beckel 1964). Similarly, some dung beetles can maintain normal rates of oxygen uptake at only 1% oxygen, while for other species this figure is closer to 2% (Holter and Spangenberg 1997).

In a detailed study measuring the metabolic response of tortricid pupae to hypoxic atmospheres, Zhou et al. (2000) found little change in metabolism until a critical oxygen concentration was reached, after which metabolic rate fell rapidly. Furthermore, this critical value was temperature dependent, being higher at elevated temperatures, consistent with greater metabolic demand. Without examining a larger number of hypoxic atmospheres and temperatures it was not possible in this study on huhu to identify critical oxygen concentrations. However, the data suggest that larvae exposed to 20°C were slightly under the critical oxygen concentration and much lower at 40°C. Insect mortality studies demonstrate that reducing the oxygen concentration also reduces thermo-tolerance thereby leading to increased mortality (Whiting et al. 1991, Yocum and Denlinger 1994, Whiting and Hoy 1997). Dentener et al. (1999) found exposure of huhu larvae to 100% nitrogen for 11 days at 20°C resulted in only 4.8% mortality, however, if the temperature was increased to 40°C, 99% of the larvae were killed in 8.3 h.

Huhu larvae recovering from exposure to anoxia at 40°C had elevated oxygen consumption which slowly declined, consistent with repaying an oxygen debt (Wegener 1993). Carbon dioxide production, however, remained relatively constant and similar to the \dot{M}_{CO_2} value for air, perhaps reflecting the observed lack of capacity to increase \dot{M}_{CO_2} at elevated temperatures. Post-anoxic recovery is facilitated by the uncoupling of circulatory and respiratory function in insects compared with vertebrates. Therefore, during recovery, an insect can immediately commence oxygen uptake through its tracheal system despite having no heartbeat and no circulation (Wegener 1993). Consequently, huhu can facilitate their recovery

by increasing oxygen consumption, whereas eliminating carbon dioxide stored in the haemolymph may be more challenging.

Conclusion

Exposure of huhu larvae to increased temperature increased \dot{M}_{O_2} until a critical temperature resulted in falling \dot{M}_{O_2} with increasing exposure time. These changes in respiratory patterns occurred between 42 and 45°C and were consistent with mortality studies on huhu exposed to heat. \dot{M}_{CO_2} reached a thermal maximum, suggesting the elimination of carbon dioxide may be a physiologically limiting factor. While the responses of small and large larvae were similar, small larvae appeared to be less thermo-tolerant to increased time-temperature exposure. RQ decreased at temperatures greater than 40°C, in contrast to the increase described in Chapter 4 during exposures between 20 and 40°C. Larval response to a temperature ramp was similar to static temperature exposure in contrast with expectations and the predictions of Neven (1998). Gas exchange in huhu still occurred at 1.8% oxygen, albeit at reduced levels, suggesting that they may use the strategy of metabolic depression to survive hypoxia. Recovery from anoxia resulted in increased oxygen consumption as larvae repaid an oxygen debt. The respiratory response of huhu larvae to stress is characterised by critical temperature and oxygen concentrations, which could be utilised in the development of commodity disinfection of insect pests.

HUHU HAEMOLYMPH pH AND LACTATE LEVELS IN RESPONSE TO STRESS

Introduction

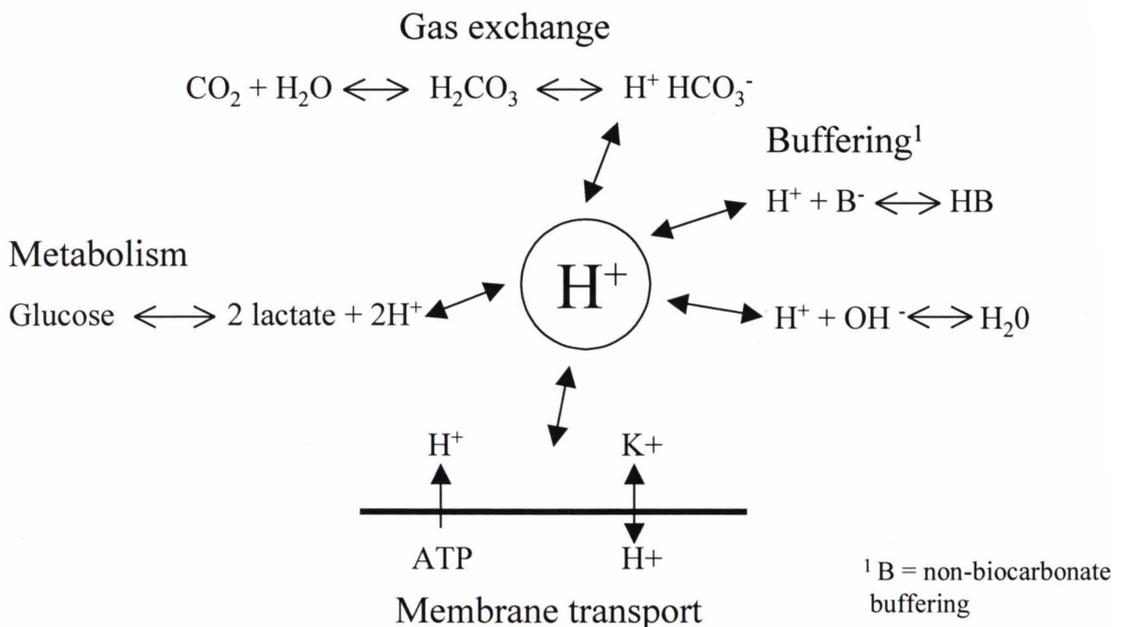
Unlike vertebrates, insect haemolymph does not function as an oxygen carrier, however, it has many other physiological functions. Haemolymph acts as a lubricant and provides structural support, which is especially important in soft-bodied larval stages. Haemolymph also has immunological functions and in some adult flying insects, is involved in thermoregulation. Circulation of the haemolymph delivers nutrients, enzymes, hormones, metabolites, waste products and water to and from the cells and tissues (Nation 2002). Haemolymph also performs a storage role for these compounds and is the insect's main reservoir of trehalose, the primary insect metabolic sugar. It also, importantly, acts as a sink for carbon dioxide, which goes into solution as the bicarbonate ion (HCO_3^-). Naturally this has implications for the acid-base status and homeostatic functioning of the haemolymph and the whole insect.

Insect haemolymph is typically slightly acidic with pH ranging between 6 and 7.5 (Levenbrook 1950, Wigglesworth 1972). Figure 6.1 shows the main physiological processes that have an impact on an organism's acid-base status (Harrison 2001). In any fluid within an organism there are equilibrium reactions linked to gas exchange, buffering, metabolism and membrane transport. Insect haemolymph pH decreases at elevated temperatures. Harrison (1988, 1989b) found that between 10 and 25°C, pH remained constant, but decreased at 0.017 pH units per °C above 25°C in two species of grasshopper. A possible mechanism for haemolymph pH change with increasing temperature is transmembrane acid-base transport (Harrison 1988). Most studies have focussed on temperatures that organisms are most likely to encounter in their natural environment. However, Ahearn (1970a) studied haemolymph properties associated with heat-killed tenebrionid beetles, where pH dropped from 6.92 to 6.36 for control (23°C) and 50°C-treated insects respectively. In an extensive study of the activity physiology in *Melanoplus bivittatus*, the two-striped grasshopper, Harrison et al. (1991) found sustained hopping caused respiratory extracellular acidosis and increased muscle lactate levels. Diet does not affect

haemolymph pH (Harrison et al. 1991); starved and fed locusts excreted net acid and base respectively with different rates of bicarbonate excretion, but still had similar haemolymph pH values (Harrison and Kennedy 1994). This provided evidence that despite major acid-base challenges, haemolymph pH remains stable, and the excretory system has a role in regulating pH.

Insects employ a range of strategies to maintain acid-base homeostasis. Haemolymph contains both bicarbonate and non-bicarbonate (mostly protein) buffer systems. In locusts, Harrison et al. (1990) demonstrated that when haemolymph P_{CO_2} was maintained at a constant level by the insect, approximately 57% of the buffer value of the haemolymph was from the bicarbonate buffer system. However, only the non-bicarbonate buffers are of physiological importance during respiratory and metabolic acidosis (Harrison et al. 1991, Harrison 2001). Elevated carbon dioxide levels in insects stimulate ventilation, indicating that the ventilatory system is actively involved in acid-base regulation, (Harrison 1989a, Gulinson and Harrison 1996). Furthermore, in locusts injected with hydrochloric acid, a pH drop of 0.5 units was completely recovered within 8-24 h, restoring normal haemolymph pH, P_{CO_2} and HCO_3^- , thereby providing the first real proof of acid-base regulation (Harrison et al. 1992). The gut and renal system of insects also plays a major role in active acid–base regulation. Phillips et al. (1994) determined that the excretory system of locusts fed or injected with acid was more than able to regulate haemolymph pH.

Figure 6.1 A simplified representation of organismal processes that contribute to acid–base physiology, after Harrison (2001).



Numerous studies indicate that insects utilise anaerobic pathways under hypoxic or anoxic conditions that result in the production of lactate (Zachariassen and Pasche 1976, Friedlander and Navarro 1979, Wegener 1993, Kolsch 2001). However, anaerobic glucose metabolism does not follow the standard pathway from glucose to lactate as seen in vertebrates; instead acetate, alanine, pyruvate and polyols are the anaerobic end products in addition to lactate (Meyer 1980). Insects typically have low levels of lactate dehydrogenase, which restricts lactate build up and associated injurious acidosis in the tissues (Wegener 1993, Weyel and Wegener 1996, Hoback and Stanley 2001). Post-anoxic recovery is generally fast in insects, aided by oxygen being able to reach the tissues via the tracheal system independent of the circulatory system (Weyel and Wegener 1996). Hypermetabolic activity follows the return to normoxia as the animal repays an oxygen debt (Wegener and Moratzky 1995, Hoback and Stanley 2001, Kolsch et al. 2002).

Chronic hypoxia reduces insect growth rates compared with normoxia (Greenberg and Ar 1996) and combined with higher temperatures, has a greater effect on development (Frazier et al. 2001, Wang et al. 2001). Greenlee et al. (1998) found that hypoxia (2 kPa P_{O2}) caused a progressive increase in haemolymph pH in locusts. This pH increase was caused by a net transfer of bicarbonate to the haemolymph and they theorised this may be due to intracellular pH regulation.

The primary objective of this study was:

8. To determine if larvae use anaerobic respiration when exposed to stress caused by heat and low oxygen atmospheres.

The aims of this study were to determine the effects of extreme temperatures (40°C), in combination with anoxic and hypoxic atmospheres, on haemolymph pH and lactate concentrations in larval huhu; the hypothesis being that increased metabolic stress would result in higher levels of anaerobiosis in huhu larvae. In an attempt to link the previous chapter's respirometry data to changes in acid-base status and lactate production, the same huhu larvae were sampled after respirometry. To determine the effect of exposure time on haemolymph pH and lactate levels, the progressive response of huhu larvae was measured at 20 and 40°C in combination with a 1.8% oxygen and a 100% nitrogen atmosphere. Metabolic

responses, such as changes in haemolymph pH and lactate levels, to these stresses may be useful in the development of commodity disinfestation treatments.

Materials and methods

Equipment

Haemolymph pH was measured using an IQ 150 pH meter equipped with an ion-sensitive, field-effect transistor (ISFeT) PH17-SS microprobe (IQ Scientific Instruments INC., San Diego, CA, USA), but also initially with a KS701 ISFeT meter (Shindengen electric MFG. Co., Japan), both with temperature compensation. Conventional glass microelectrodes were found to be unsatisfactory, having a slow response time and subject to clogging when exposed to haemolymph protein. Prior to use and as required, calibration was carried out using BDH colour-key buffers, pH 4.0 and 7.0 ± 0.02 (BDH Laboratory Supplies, UK). A Shimadzu UV1601 spectrophotometer (Shimadzu Corporation, Japan) was used for lactate determination.

Elevated temperature

Haemolymph samples for measurement of pH and lactate, were taken 10 min after huhu were removed from the water bath (post-respirometry, Chapter 4 and 5) to allow them to cool to ambient temperature (19-21°C). Measurements were also conducted on two control groups of large huhu ($n = 16$ for each group, starved overnight). One group were sampled immediately (time = 0) while the other group were sampled after 5 h exposure to humidified (saturated) air at 20°C flowing at 25-30 sccm (standard cubic centimetres per min) through 10 ml disposable syringes, which contained the huhu larvae. Each sample of haemolymph was carefully withdrawn from the dorsal blood vessel of the 6th abdominal segment using a 0.5 ml low dead-space disposable tuberculin syringe (Monoject, Sherwood Medical, St. Louis, MO). Measurements of pH were made with a minimum of 10 μ l of haemolymph but larger volumes were used when extraction was straightforward and insect size permitted. Haemolymph was deproteinated by adding 10 or 20 μ l of haemolymph to either 20 or 40 μ l of 8% perchloric acid for “small” or “large” huhu respectively, prior to storage at -18°C for subsequent lactate analysis.

Hypoxic and anoxic atmospheres

Response after respirometry

Measurements of haemolymph pH and lactate sampling were conducted on huhu after respirometry under hypoxic conditions as described in Chapter 5. Briefly, huhu larvae were either exposed to pure nitrogen for one hour (55.5 min plus 5 minutes purging with air) followed by 4 h of respirometry with normal baseline air,

or 1.8% oxygen for 5 h. Comparisons were made with huhu larvae after respirometry at 20°C with dry air.

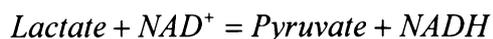
Progressive response

“Large” size huhu larvae (3-4 g, starved overnight as described in Chapter 3) were contained in 10 ml disposable syringes and exposed to either air, 1.8% oxygen (1.78% oxygen balance nitrogen) or pure nitrogen (BOC Gases NZ Ltd). Each syringe was supplied with gas at 25-30 sccm, via a 16-way aluminium and copper manifold, after first being saturated by bubbling through a water-filled Drechsel bottle (Quickfit MF29/3/500, England). The system was connected with flexible Tygon[®] tubing (Norton Performance Plastics, Akron, OH, USA) and the entire apparatus was immersed in a water bath (Grant Instruments, Cambridge, UK). Experiments with air were conducted at 20°C and huhu were removed from syringes after 0 and 5 h. Larvae subjected to 1.8% oxygen or 100% nitrogen were exposed to 20 and 40°C and sampled after 0.5, 1, 2.5, and 5 h. Larval haemolymph pH was measured, and a sample collected for lactate analysis as described above. Larvae exposed to 40°C were allowed to cool to ambient whereas larvae were sampled immediately after 20°C treatments. Larvae were not placed back in the system after measurement, therefore each sample represents an independent measure (n = 8 huhu per sample time and 16 for the controls, spread over 2 d, n = 32 per treatment).

Mortality data were recorded 3 d after treatment by storing unfed larvae at 20°C; they were examined by prodding and deemed to be alive if any movement was observed.

Determination of Lactate

Lactate was determined using a micro-version of the Sigma enzymatic lactate procedure 826A-UV (Sigma Diagnostics, St Louis, MO, USA). In this procedure, lactate dehydrogenase (LDH) catalyses the following reaction:



where NAD^+ is β -nicotinamide adenine dinucleotide and NADH is β -nicotinamide adenine dinucleotide, reduced form. Deproteinated samples were centrifuged at 7,500 g for 5 minutes, 10 μl of the supernatant was removed and added to 400 μl of the test reagent (comprising 834 $\text{mg}\cdot\text{L}^{-1}$ NAD, 0.2 M glycine buffer (pH 8.96) and

8340 IU.L⁻¹ of LDH). Samples were incubated at 37°C for 1 hour then read at 340 nm. The spectrophotometer was calibrated using 0, 0.89, 2.22 and 4.44 mM lactate standards (mean of n = 3 standards at each concentration) and the resulting relationship of absorbance to lactate concentration was used to calculate sample lactate levels.

Statistics

Data were analysed using analysis of variance, one or two-way as appropriate, on response data, or Log₁₀ (x+1) in the case of lactate to standardise residual errors using either Minitab® or Genstat (Release 14, Minitab Inc., USA; Genstat V.7.2, VSN International Ltd, UK). Means were compared for significance ($P < 0.05$) using either Tukey's HSD or through calculation of an LSD using the Student's t-test. Regression lines were fitted and plotted using Origin™ (Version 5.0, Microcal Software Inc., MA, USA).

Results

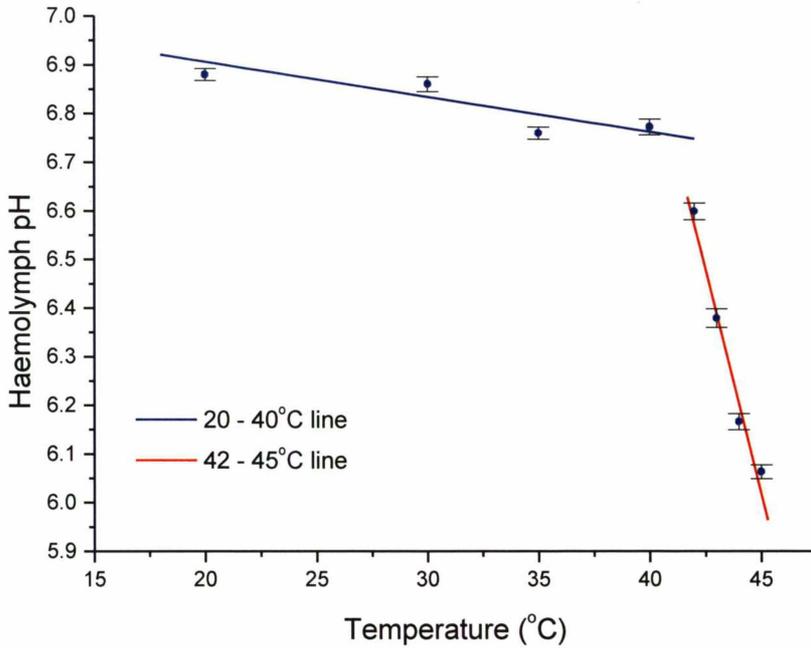
Elevated temperature

ANOVA of huhu haemolymph pH values after respirometry at different temperatures indicated that larval size was not a significant factor ($F = 0.02$, $P = 0.89$) in contrast to temperature, which was strongly significant ($F = 334$, $P < 0.001$), therefore both size classes were combined for plotting. Haemolymph pH declined slightly between 20 and 40°C (slope = -0.0071 ± 0.0011 (\pm se)), but decreased steeply between 42 and 45°C (slope = -0.185 ± 0.009 (\pm se)), Figure 6.2A). To enable comparisons to be made with additional large control larvae at 20°C, only large-size data were analysed further. Larvae exposed to 43-45°C had pH values significantly lower compared with lower temperatures and also significantly different to each other ($P = 0.05$, Tukey's pair-wise comparisons, Table 6.1). There was no significant difference between huhu haemolymph pH values of larvae exposed to dry air at 20 and 30°C for 16 and 5 h respectively. However, starved huhu that did not undergo respirometry (20°C time = 0) had a significantly lower pH, but larvae that were contained for 5 h (20°C humidified) were not significantly different to all treatments less than 42°C ($P = 0.05$, Tukey's pair-wise comparisons).

Huhu haemolymph lactate concentrations were ≤ 1 mM below 40°C and then increased between 42 and 44°C (depending on size) before levelling off at 44-45°C (Figure 6.2B and Table 6.1). There were differences in response due to huhu size ($F = 4.3$, $P = 0.014$) and temperature ($F = 100$, $P < 0.001$) on $\text{Log}_{10}(x + 1)$ transformed data. Analysis at 44°C demonstrated that small huhu larvae had significantly more lactate produced in their haemolymph compared to large, with mean values of 6.97 and 5.29 mM respectively (ANOVA $F = 5$, $P = 0.031$). As with the pH data there were some small significant differences between some of the controls and the lower temperatures (Table 6.1).

Figure 6.2 Change in haemolymph pH of huhu larvae after 5 h respirometry at static temperatures (error bars indicate SEM). (A) Mean pH regression equations for 20-40°C and 42-45°C respectively are $\text{pH} = -0.0071\text{Temperature} + 7.05$, $r^2 = 0.19$, $P < 0.0001$; $\text{pH} = -0.185\text{Temperature} + 14.3$, $r^2 = 0.74$, $P < 0.0001$. (B) Mean lactate concentration.

A.



B.

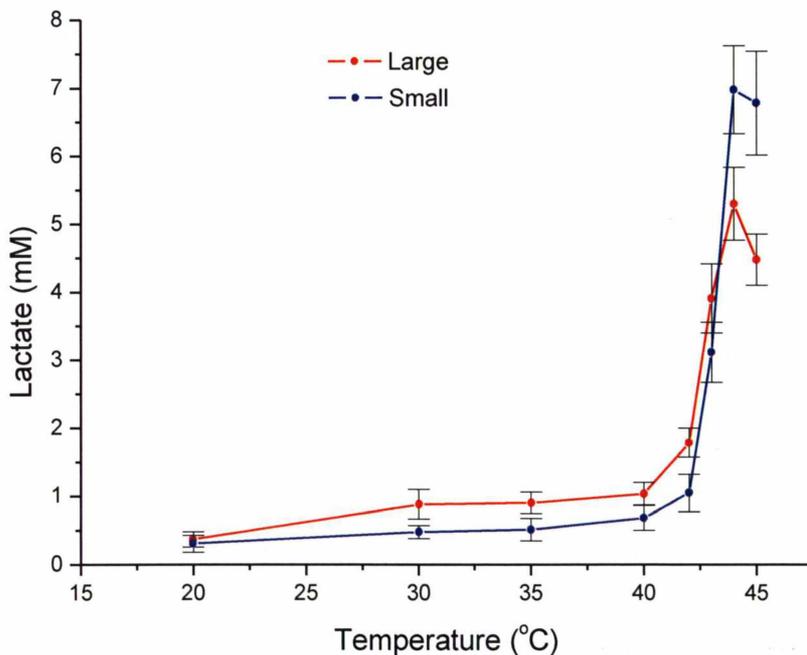


Table 6.1 Mean haemolymph pH and lactate concentrations of large huhu larvae after respirometry at 20-45°C with dry air. Additional measurements on control huhu not exposed to respirometry (time = 0) and contained with a humidified airflow for 5 h, both at 20°C, are included for comparison. Means within columns not followed by the same letter are significantly different (Tukey's pair-wise comparisons $P < 0.05$).

	Mean pH \pm SEM	Mean lactate (mM) \pm SEM	n
20°C time = 0	6.75 \pm 0.032 b	0.59 \pm 0.076 ab	16
20°C humidified	6.77 \pm 0.024 ab	0.82 \pm 0.075 a	16
20°C	6.87 \pm 0.023 a	0.37 \pm 0.112 a	21
30°C	6.88 \pm 0.030 a	0.88 \pm 0.219 ab	21
35°C	6.71 \pm 0.021 bc	0.90 \pm 0.156 ab	21
40°C	6.76 \pm 0.027 ab	1.04 \pm 0.165 bc	14
42°C	6.62 \pm 0.024 c	1.78 \pm 0.211 c	20
43°C	6.39 \pm 0.027 d	3.80 \pm 0.509 d	28
44°C	6.20 \pm 0.020 e	5.29 \pm 0.537 d	28
45°C	6.07 \pm 0.021 f	4.47 \pm 0.375 d	14

Hypoxic and anoxic atmospheres

Response after respirometry

Haemolymph pH levels were all significantly reduced after exposure to nitrogen at 40°C (1 h followed by 4 h recovering in air) and 1.8% oxygen at 20 and 40°C compared with normal air at 20°C (Table 6.2; ANOVA $F = 84.5$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.05$). Lactate concentrations in huhu haemolymph increased to 4.5 mM after exposure to 1.8% oxygen at 40°C. This was significantly different to all other temperature and atmosphere combinations and also corresponded to the greatest reduction in pH. There was no significant difference in lactate levels between recovery from nitrogen exposure and air at 20°C indicating any accumulated lactate from exposure to nitrogen had been metabolised. The overall trend was one of increasing haemolymph acidosis and lactate, with both elevated temperature and reduced oxygen concentration, with both combined causing the greatest change. Despite significant differences in both pH and lactate concentration at 1.8% oxygen and 20°C, compared with normoxia at 20°C the

magnitude of difference was small, yet within minutes of being exposed to 1.8% oxygen all movement ceased, which contrasted greatly with normoxia.

Table 6.2 Mean haemolymph pH and lactate concentrations of large huhu larvae after 5 h of respirometry at 20 and 40°C with a 1.8% oxygen atmosphere and 40°C recovery from pure nitrogen (55 min N₂ followed by 5 min normal air). Measurements on huhu after respirometry at 20 and 40°C are included for comparison. Means within columns not followed by the same letter are significantly different (Tukey’s pair-wise comparisons $P = 0.05$).

	Temperature °C	Mean pH ± SEM	Mean lactate (mM) ± SEM	n
N ₂ recovery	40°C	6.52 ± 0.027 c	0.47 ± 0.089 ab	14
1.8% O ₂ ,	20°C	6.74 ± 0.018 b	0.83 ± 0.099 b	14
1.8% O ₂ ,	40°C	6.32 ± 0.024 d	4.54 ± 0.387 c	14
Air,	20°C	6.87 ± 0.023 a	0.37 ± 0.112 a	21
Air,	40°C	6.76 ± 0.027 b	1.04 ± 0.165 b	14

Progressive response

The effects of atmosphere (air, nitrogen or 1.8% oxygen) and temperature (20 and 40°C) on haemolymph pH were both significant after 300 min ($F = 190, 270$ respectively and $P < 0.001$). However, the interaction of atmosphere and temperature was not significant ($F = 2.07, P = 0.16$) indicating the effect of atmosphere on pH does not differ with temperature and vice versa. Mean huhu haemolymph pH of large larvae decreased during exposure to nitrogen and 1.8% oxygen at 40°C (Table 6.3). There was no significant difference in the mean control pH values at time = 0 and 300 min ($F = 0.24, P = 0.63$). However, the pH of larvae exposed to 1.8% oxygen at 20°C, time = 30 min, was significantly higher compared to the time = 0 control (Table 6.3, Figure 6.3A). After 300 min, all treatments were significantly lower than the control with the exception of 1.8% oxygen at 20°C. The greatest reduction in pH occurred with exposure to nitrogen at 40°C. The overall trend indicated that reducing the oxygen concentration, and or increasing the temperature with increased exposure time to these stresses, reduced haemolymph pH.

Table 6.3 Mean haemolymph pH of large huhu larvae exposed to 1.8% oxygen and pure nitrogen at 20 and 40°C after 30 and 300 min, compared with untreated control larvae (zero time) and larvae caged for 300 min in air at 20°C. Means within columns not followed by the same letter are significantly different (Tukey's pair-wise comparisons $P = 0.05$).

	Mean pH ± SEM		n
	Elapsed time = 30 min	Elapsed time = 300 min	
Control, air 20°C	¹ 6.75 ± 0.032 ab	6.77 ± 0.024 c	16
1.8% O ₂ , 20°C	6.98 ± 0.028 c	6.83 ± 0.031 c	8
1.8% O ₂ , 40°C	6.77 ± 0.029 a	6.37 ± 0.023 b	8
N ₂ , 20°C	6.86 ± 0.045 bc	6.46 ± 0.021 b	8
N ₂ , 40°C	6.62 ± 0.052 a	5.91 ± 0.037 a	8

¹ Elapsed time was 0 for the control

The effects of atmosphere and temperature on haemolymph lactate concentrations were both significant after 300 min ($\log_{10}(x+1)$ transformed response, $F = 253$, 79 respectively and $P < 0.001$). However, the interaction of atmosphere and temperature was just significant ($F = 4.24$, $P = 0.046$) indicating the effect of atmosphere on lactate differs with temperature and vice versa. There was no significant difference in the mean control lactate concentrations at time = 0 and 300 min ($F = 2.82$, $P = 0.10$). After 30 min, all treatments showed significantly increased lactate levels compared to time = 0 control (Table 6.4, Figure 6.3B). Nitrogen at 40°C caused significantly greater lactate to be produced than the three less severe treatments at this time. After 300 min, all treatments were significantly higher than the control with the exception of 1.8% oxygen at 20°C, which remained low throughout the experiment. At this time, huhu treated with nitrogen at 40°C had a 23-fold increase in lactate production. Overall lactate production in response to temperature and atmosphere stresses had the same pattern as pH; that is, a decrease in pH corresponding to a rise in lactate production. The only exception was 1.8% oxygen at 40°C and nitrogen at 20°C that had the same lactate response until 300 min where the values diverged and were reversed compared to the pH response.

All larvae exposed to 1.8% oxygen at 20 and 40°C were alive 3 days after treatment, as were those treated with nitrogen at 20°C. However, exposure to nitrogen at 40°C for 300 min resulted in complete mortality, 150 min exposure led to sick-looking larvae, while shorter exposures produced no effect.

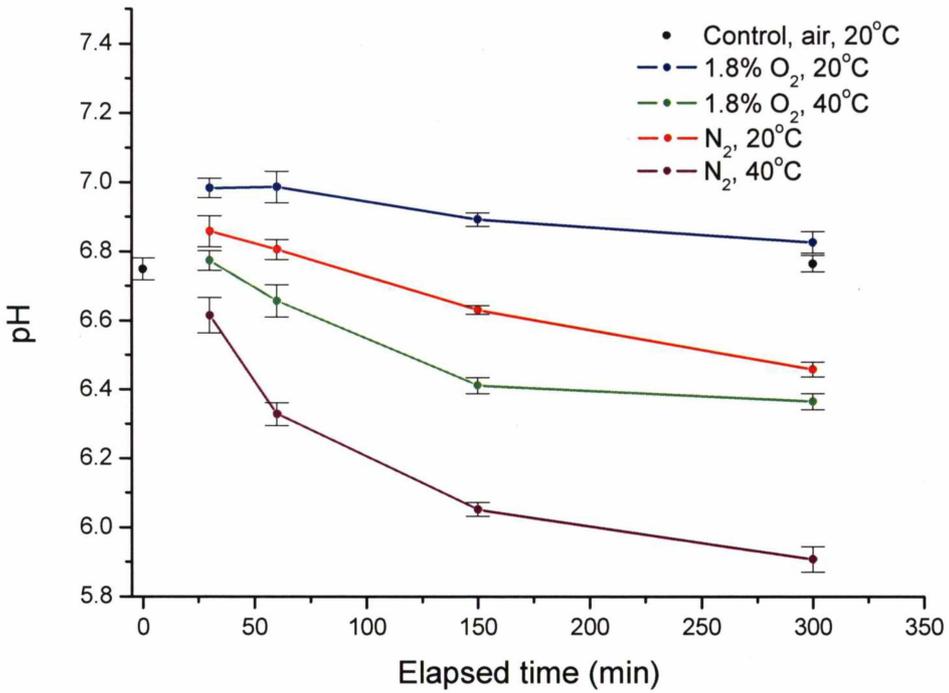
Table 6.4 Mean haemolymph lactate concentrations of large huhu larvae exposed to 1.8% oxygen and pure nitrogen at 20 and 40°C after 30 and 300 min, compared with untreated control larvae (zero time) and larvae caged for 300 min in air at 20°C. Means within columns not followed by the same letter are significantly different (Tukey's pair-wise comparisons $P = 0.05$).

	Mean lactate (mM) \pm SEM		n
	Elapsed time = 30 min	Elapsed time = 300 min	
Control, air 20°C	¹ 0.64 \pm 0.068 a	0.82 \pm 0.075 a	16
1.8% O ₂ , 20°C	1.34 \pm 0.25 b	1.13 \pm 0.19 a	8
1.8% O ₂ , 40°C	1.85 \pm 0.11 b	5.12 \pm 0.87 b	8
N ₂ , 20°C	1.47 \pm 0.26 b	9.20 \pm 0.69 c	8
N ₂ , 40°C	5.82 \pm 0.39 c	18.6 \pm 1.74 d	8

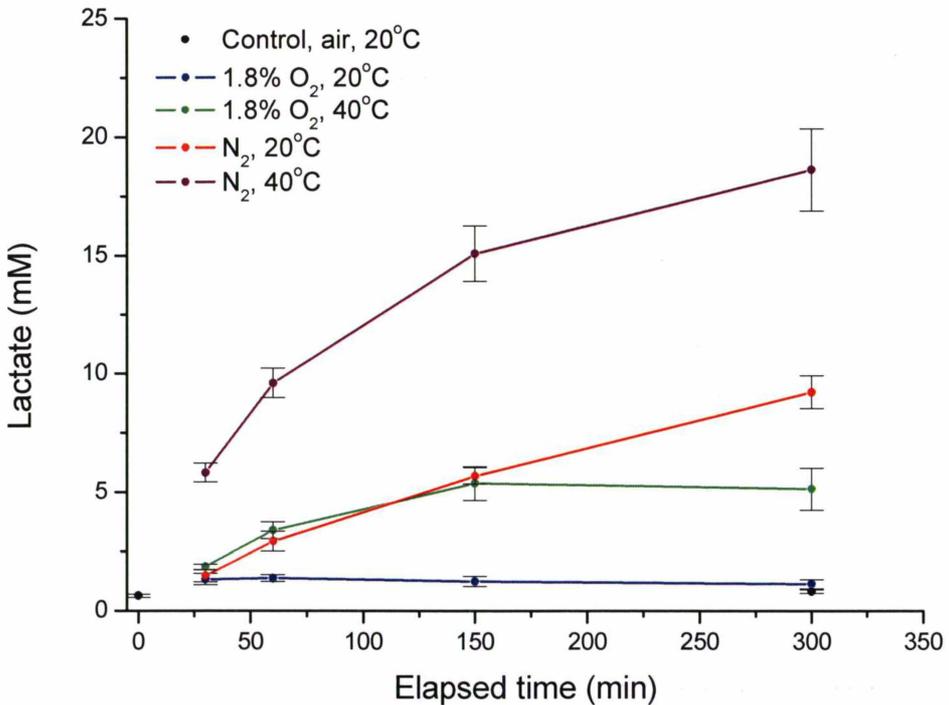
¹Elapsed time was 0 for the control

Figure 6.3 Change in haemolymph of large huhu larvae exposed to 1.8% oxygen and nitrogen atmospheres, error bars indicate SEMs, n = 8 huhu per sample (16 for the control). (A) Mean pH. (B) Mean lactate concentration.

A.



B.



Discussion

Harrison (2001) highlighted a number of important issues associated with accurate pH measurement of insect haemolymph in reviewing insect acid-base physiology. Insect haemolymph should be sampled and measured quickly to prevent loss of carbon dioxide and the subject and apparatus should be at the same temperature. Furthermore, haemolymph can clot and oxidation of phenolic compounds can lead to acidification (Harrison 1988). The advantage of using an ISFeT probe for pH measurement in this study was that it had a much faster response time (usually a few seconds) compared with conventional glass microprobes. Fortunately, huhu haemolymph did not clot even after several hours, and the pH remained stable for at least 5 min (100 μ l in a 0.5 ml microcentrifuge tube).

Huhu larvae exposed to elevated temperatures were allowed to cool to ambient temperatures for 10 min prior to sampling. While this ensured the pH probe and animal were at the same temperature, it potentially leaves the method open to several criticisms. Firstly, it allowed time for some recovery from acidosis at ambient temperature and atmospheric conditions, and secondly, in vitro effects of temperature on haemolymph have been recorded. Harrison (1988) found in vitro changes to be less than -0.004 pH unit per $^{\circ}$ C in the two-striped grasshopper, which for a 25 $^{\circ}$ C drop in temperature (45 to 20 $^{\circ}$ C) equates to a rise of 0.1 of a pH unit. Therefore the measurements in this study may underestimate the true reduction in haemolymph pH caused by elevated temperature exposure. However, the overall trend was unaffected in light of a 0.8 pH unit change between 20 and 45 $^{\circ}$ C exposures. After exposure to such extreme temperatures for up to 5 h, huhu larvae would have experienced varying levels of irreversible physical damage that would probably have limited the insects' ability to recover. Exposure to lower temperatures and temperature-atmosphere combinations, such as 1.8% oxygen at 40 $^{\circ}$ C were more likely to have been affected by recovery.

Elevated temperature

Huhu haemolymph pH after respirometry followed a biphasic decline (Figure 6.2A) with increasing temperature. The first phase of the decline between 20 and 40 $^{\circ}$ C was gradual with a slope of -0.0071 ± 0.0011 (\pm SE), which was similar to that reported by Birchard et al. (1991) for *Manduca sexta* (Lepidoptera), and falls

between the in vitro and in vivo values for the two-striped grasshopper (Harrison 1988). However, the decline at temperatures greater than 40°C was much steeper (slope -0.185 ± 0.009 (\pm SE)) and corresponds to changes in \dot{M}_{O_2} , \dot{M}_{CO_2} (see Chapter 5) and lactate. It is tempting to speculate that catabolism was simply being pushed so hard that gas exchange was unable to keep up, leading to respiratory acidosis and lactate production (metabolic acidosis). While this respiratory stress may be a significant component of the measured pH changes, these huhu larvae were in the process of dying, and therefore would have experienced cellular death that may also lead to acidosis. Considering that lactate production closely accompanied the decrease in pH confirms a significant anaerobic component to metabolism in heat-treated huhu larvae.

Ahearn (1970a) found haemolymph pH in *Centrioptera muricate*, a desert tenebrionid, dropped from to 6.92 at 23°C to 6.69 when exposed to 47°C for 1 h. All beetles died, as defined by loss of movement after 50 min at 47°C, whereas at 45°C, beetles continued to move throughout the 1 h exposure. The exposure of these beetles to 47°C also resulted in a modest 2.6-fold increase in haemolymph lactate but this increased to a 6.5 fold increase after exposure to 55°C. Similarly, the haemolymph pH dropped to 6.36 at 50°C. Huhu larvae had a 12-fold increase in lactate at 45°C after respirometry compared with 20°C. Considering this tenebrionid is adapted to desert conditions, and continued to move at 45°C, it was not surprising it displayed greater heat tolerance and had smaller haemolymph pH and lactate changes at similar temperatures.

At high temperatures, small huhu produced more lactate compared to large ones. This was consistent with their metabolic response described in Chapter 5. Overall, small larvae had a higher specific metabolic rate (\dot{M}_{O_2}), and a greater decline in \dot{M}_{O_2} with increasing temperature and time, compared to larger larvae. This suggests that small larvae compared with large ones, were exposed to greater metabolic demand caused by increased temperature, which led to greater stress and hence increased anaerobiosis. However, haemolymph pH was statistically the same for both small and large larvae, whereas greater acidosis could be expected with small huhu. There may be differences in the pH buffering capacity of small and large larvae which could account for this, although without an in-depth investigation of huhu haemolymph biochemistry any possible explanation would be speculative.

There were some differences between haemolymph control (20°C) values. Larvae subjected to respirometry with dry air, had slightly higher pH values than those measured directly from a ventilated Petri dish and those exposed to humidified air for 300 min. It was impossible to distinguish the part that containment, dry air, and different amounts of handling contributed to these differences, but they were slight in relation to the overall measured responses.

Hypoxic and anoxic atmospheres

Response after respirometry

Reduced haemolymph pH and increased lactate in huhu after exposure to extreme temperatures and/or reduced oxygen atmospheres during respirometry was consistent with the general hypothesis of this study. Hypoxia (1.8% O₂) at 20°C resulted in a slight decrease in pH and increase in lactate. In contrast, Greenlee and Harrison (1998) found hypoxia caused the haemolymph of grasshoppers to become more alkaline. However, these grasshoppers were exposed to different atmospheres for shorter periods, compared to the animals in this study; also they were a different life-stage and order. Data from the progressive response studies indicated exposure of huhu larvae to nitrogen at 40°C for 1 h led to a significant reduction in pH by 0.4 units and a nearly 10-fold increase in lactate. Therefore, prior to normoxia commencing during the nitrogen-recovery-respirometry described in Chapter 5, larvae would have had similar levels of acidosis and lactate. Haemolymph pH failed to return to normal during a 4 h period of normoxia to recover from anoxia. In contrast, lactate levels were normal, suggesting recovery of lactate was not coupled with recovery of pH. This may be linked to higher post-anoxic \dot{M}_{O_2} , and a constant \dot{M}_{CO_2} described in Chapter 5. The ability of huhu to recover from anaerobiosis may be linked to the modified atmospheres they encounter within logs.

Progressive response

The overall hypothesis of increased anaerobiosis was confirmed by changes in huhu acidosis and lactate levels, which increased with the severity of the treatment and exposure time. However, larvae exposed to 1.8% oxygen at 20°C had significantly more alkaline haemolymph after 30 min exposure, which was consistent with the findings of Greenlee and Harrison (1998), although after 300 min this treatment was not significantly different to the control. Larvae exposed to 1.8% oxygen at 20°C ceased to move and had reduced gas exchange (reduced CO₂ production), thereby indicating they were experiencing metabolic stress because the oxygen

concentration had dropped below some critical threshold (Portner and Grieshaber 1993, Wegener and Moratzky 1995, Weyel and Wegener 1996, Zhou et al. 2000).

The patterns of huhu haemolymph pH decreasing and lactate increasing over time were very similar for the different treatments. An exception to this trend was 1.8% oxygen at 40°C, which had lower lactate levels considering its pH response. This relationship between pH and lactate was consistent with acidosis associated with anaerobic respiration (Friedlander and Navarro 1979, Harrison et al. 1991, Wegener 1993, Greenlee and Harrison 1998, Kolsch 2001, Kolsch et al. 2002, Nation 2002). While lactate is not the only anaerobic end product produced by insects, it is commonly detected after exercise, exposure to hypoxic or anoxic conditions, and elevated carbon dioxide atmospheres (Harrison et al. 1991, Harrison and Kennedy 1994, Phillips et al. 1994, Greenlee and Harrison 1998). In huhu, lactate was clearly a major anaerobic end product. This contrasts with the general view that insects have low levels of lactate dehydrogenase, which restricts lactate build up and associated injurious acidosis in the tissues (Wegener 1993, Weyel and Wegener 1996, Hoback and Stanley 2001). In *Ephestia cautella* (Lepidoptera), Navarro and Friedlander (1975) found haemolymph lactate levels increased 48-fold upon exposure to a 1% oxygen atmosphere. This insect like huhu has an atypical anaerobic response to hypoxia. Furthermore, this insect is a stored-product pest and therefore is naturally exposed to a hypoxic environment, similar to that of huhu. Clearly, increased temperature combined with hypoxia and anoxia exposed the huhu larvae to greater stress, as reflected by haemolymph pH and lactate. The exposure time to this stress was also important (Figure 6.3 A and B) and reflected by observations of mortality where the longest exposure to nitrogen at 40°C resulted in complete mortality.

Conclusions

Temperatures greater than 40°C with normoxic atmospheres caused huhu larvae to increasingly utilize anaerobic metabolic pathways. Exposure to 1.8% oxygen at 20°C was below the critical aerobic threshold for huhu larvae but did not lead to increased anaerobic respiration, therefore implying the strategy of metabolic rate depression. In contrast, exposure to anoxia resulted in anaerobic respiration, most probably accompanied by metabolic depression. Responses to anoxia and hypoxia were both amplified by elevated temperature and increasing exposure time. However, to determine the underlying biochemical mechanisms associated with

these responses requires more detailed investigation of acid-base physiology, metabolic pathways and a wider range of environmental stress conditions beyond the scope of this study.

In summary, combinations of increased temperature and reduced oxygen atmospheres with increasing exposure time resulted in increased anaerobiosis, as evidenced by increased haemolymph acidosis and lactate. Anaerobic responses in *huhu* were consistent with stress conditions, which caused changes in respiration described in Chapter 5. Clearly the primary objective of this study has been achieved. These significant responses at critical thresholds may be very useful in the development of commodity insect disinfestation methods.

GENERAL DISCUSSION

Metabolic rate

Huhu larvae can survive without food for several years, move very slowly and have a long life cycle (Morgan 1960, Edwards 1961a), which suggests they may have a low metabolic rate. However, SMR in huhu, while lower than the predicted value, was similar to other non flying coleopterans (Reinhold 1999, Lighton et al. 2001). Comparisons of huhu metabolism with other cerambycid larvae were not possible due to the scarcity of relevant studies.

Manometric respirometry was accurate and the components required were inexpensive. However, this method required a considerable amount of time recording data, and prevented the measurement of \dot{M}_{O_2} and \dot{M}_{CO_2} on the same animal at the same time, and therefore, accurate measurement of the RQ. In contrast, the automated respirometry system utilising gas analysers, was efficient, allowed simultaneous measurement of \dot{M}_{O_2} and \dot{M}_{CO_2} , but was more expensive. Both methods used closed respirometry, which has been criticised for its poor temporal resolution, and thereby allowing animal movement to affect metabolism (Lighton and Fielden 1995, Lighton et al. 2001). However, in this study these issues were addressed by observation of movement, selection of low stable readings and the use of a movement detection system.

Temperature stress

At temperatures less than 40°C, huhu metabolism increased with temperature with a mean Q_{10} of 1.69 for oxygen consumption. This metabolic response was consistent with other data for insects (Lighton and Fielden 1995, Lighton et al. 2001) and, indeed, other organisms (Gillooly et al. 2001, Clarke and Fraser 2004). Superficially there were no apparent stress responses reflected in the metabolic data. However, measuring both \dot{M}_{CO_2} and \dot{M}_{O_2} enabled calculation of the RQ, which

increased with temperature, indicative of a change in metabolic substrate. Corroborating these metabolic stress responses was the cessation of movement by larvae at about 35°C. Furthermore, when huhu were reared at 25°C compared with 20°C, larvae at the higher temperature initially developed much faster, but ultimately, development slowed and a large proportion died. This illustrates the difference between acute and chronic exposure to temperature stress.

When huhu larvae were exposed to extreme temperatures (greater than 40°C), there were obvious metabolic responses to stress at critical temperatures, as indicated by changes in the slope of the relationship between gas exchange and exposure time. This poses some fundamental questions; was this an inability of huhu to adequately respond to higher metabolic demand, metabolic depression to conserve fuel, or the result of thermal wounding (Denlinger and Yocum 1998)? Probably it was a combination of several processes. Neven (1998) found that codling moth died very soon after metabolism (\dot{M}_{CO_2}) began to decline, when they were exposed to heat ramps, even if they were returned to normal temperatures. This suggests that heat had caused systematic cell death in these insects. In huhu larvae, fat metabolism was supplemented by other substrates, such as carbohydrate, as metabolic demand increased with temperature. However, above 40°C, RQ typically decreased, which is compatible with all three theories outlined above. When huhu larvae were subjected to a temperature ramp, \dot{M}_{CO_2} peaked prior to \dot{M}_{O_2} . This supported the pattern observed at constant temperatures, where larvae reached a maximum \dot{M}_{CO_2} level with increasing temperature, in contrast to \dot{M}_{O_2} which continued to increase. This metabolic response suggests huhu could not respond to the increased metabolic demands of higher temperatures, which could lead to cellular death. Alternatively, thermal wounding could directly prevent the elimination of carbon dioxide via the tracheal system.

Haemolymph acidosis and lactate dramatically increased in larvae exposed to temperatures greater than 40°C, corresponding to the observed changes in gas exchange. Both acidosis and lactate accumulation are consistent with anaerobic respiration (Friedlander and Navarro 1979, Harrison et al. 1991, Kolsch 2001, Kolsch et al. 2002). However, the physiological processes that may have led to these biochemical changes in huhu haemolymph cannot be determined from this study.

Reduced oxygen

Gas exchange continued during exposure of huhu larvae to a hypoxic atmosphere of 1.8% oxygen at 20°C. \dot{M}_{CO_2} was slightly lower at this temperature (not significant), and haemolymph pH and lactate remained close to control levels. However, larvae were clearly experiencing stress as they ceased to move, consistent with the strategy of metabolic depression (Wegener and Moratzky 1995, Weyel and Wegener 1996). When the temperature was increased to 40°C with 1.8% oxygen, there was a decrease in pH and increase in lactate compared with both 20°C and 40°C air exposure. This finding was consistent with a reduction in \dot{M}_{CO_2} at 40°C with 1.8% oxygen compared with air at the same temperature, and therefore a greater level of metabolic depression in response to a more severe stress. However, at low oxygen atmospheres, gas exchange is not equivalent to metabolism, which can be supplemented by anaerobic pathways.

Exposure of huhu larvae to anoxia (pure nitrogen) was clearly more stressful than 1.8% oxygen, as evidenced by higher levels of lactate and greater reductions in pH within the haemolymph. Respirometry after 55 min of anoxia at 40°C resulted in higher levels of \dot{M}_{O_2} , consistent with the repayment of an oxygen debt incurred while larvae utilised anaerobic pathways. Interestingly, \dot{M}_{CO_2} was not increased and remained below the level measured for air at 40°C for the remainder of the experiment. Failure to increase \dot{M}_{CO_2} , and eliminate carbon dioxide stored in the haemolymph, probably resulted in the reduction in haemolymph pH measured at the conclusion of the experiment. In contrast, larval lactate levels had returned to normal. These findings are consistent with the uncoupling of respiratory and circulatory function in insects, where oxygen is immediately available via the tracheal system during recovery under normoxic conditions (Wegener 1993). Adaptations of huhu larvae to their hypoxic environment within logs probably enable them to survive and recover from hypoxia. Their ability to utilise anaerobic pathways in response to low oxygen stress is not typical of many insect species (Wegener 1993), and may be related to the larval environment within logs. The stored-product pest *Ephestia cautella* also inhabits hypoxic environments, and produces high levels of lactate when exposed low oxygen stress (Navarro and Friedlander 1975).

Exposure time

The magnitude of the response to stress in huhu larvae was always influenced by time. Exposure time was significant in all respirometry studies at extreme temperatures as well as the interaction of time and temperature. Time was also a determinate of haemolymph acidosis and lactate stress responses to hypoxic or anoxic-temperature treatments. Furthermore, the severity of the stress of anoxia at 40°C was highlighted by complete mortality of the huhu larvae after 300 min exposure, whereas after 150 min exposure to the same treatment, larvae looked sick but none were dead (all larvae assessed 3 d post-treatment). These findings are consistent with mortality studies on insects and have led several researchers to develop time-temperature mortality models (Nedved et al. 1998, Tang et al. 2000, Hansen et al. 2004).

Relevance

This research provides detailed information on how huhu larvae respond to thermal stress. Likewise, investigation of selected, reduced oxygen-temperature treatments indicated a high level of anaerobiosis in larvae. However, elucidating the physiological processes causing these responses requires far more investigation than the scope of this study allows. The respiratory responses and increased anaerobic respiration found in this study, when an insect is exposed to stress, may assist the development of commodity disinfestation protocols. This is contingent on the assumption that higher levels of stress, which cause greater physiological responses, are linked to increased mortality. This assumption not only seems reasonable, but is supported by the findings of Neven (1998) who found changes in respiration with temperature were correlated to codling moth mortality. Zhou (2001) measured the metabolic heat rate (MHR, measured by calorimetry) of a tortricid pest exposed to different reduced oxygen and elevated carbon dioxide combinations. This study found complete mortality of pupae was achieved when the MHR dropped below 50%. While there was no direct correlation between MHR and treatment severity, these studies provided insight into the mode of action of controlled atmospheres on this insect.

Physiological data on candidate insect pests could be used to underpin disinfestation research in a number of ways, including such research components as determining the most efficacious treatment combinations (Zhou et al. 2000), the most resistant life-stage, and comparing species tolerance. Detailed investigation of insect

physiological and biochemical responses to disinfestation stress can provide vital information on the mode of action of such stresses (Carpenter and Potter 1994, Neven 2000, Zhou et al. 2001). For example, Lester and Greenwood (1997) found exposure of the leafroller pest, *Epiphyas postvittana*, to sublethal temperatures induced thermo-tolerance to a normally fatal heat treatment. Furthermore, this thermo-tolerance was correlated with the induction of heat shock proteins. Thermo-tolerance of disinfestation pests can be modelled and has been used in the development of disinfestation treatments (Neven 1998, Waddell et al. 2000).

A major advantage of using physiological data to supplement mortality data is the increased efficiency gained by the requirement of a much smaller number of insects. Detailed and statistically robust physiological data can be generated from relatively few insects, compared to the vast numbers required to predict mortality, therefore enabling disinfestation treatments to be developed quicker and for reduced cost (Carpenter and Potter 1994).

Future research

The effects of a wider range of reduced oxygen atmospheres on the metabolic response of huhu would provide precise identification of critical values (Portner and Grieshaber 1993, Zhou et al. 2000). This could be extended to include the effects of elevated levels of carbon dioxide, which can be more toxic to some insects than low oxygen (Carpenter and Potter 1994, Zhou et al. 2000, 2001). Calorimetry or respirocalorimetry may be more useful than respirometry because it measures MHR, which includes anaerobic processes (Acar et al. 2001, Downes et al. 2003). Further investigation of the relationship between physiological stress and mortality in huhu would be beneficial.

Detailed investigation of insect haemolymph biochemistry during exposure to temperature and CA stress may provide a better understanding of the mode of action of these stresses. Measurements of lactic dehydrogenase (Navarro and Friedlander 1975), dissolved carbon dioxide, buffering capacity (Harrison 2001), and haemolymph circulation (Wasserthal 1996) in real time would be especially useful.

In Summary

Huhu larvae experienced thermal stress with chronic exposure to temperatures as low as 25°C. Acute exposure of larvae up to 40°C resulted in incremental increases

in their metabolic rate, changes in metabolic substrate, and cessation of movement at about 35°C. Temperatures greater than 40-42°C led to a time-temperature-dependent decline in metabolic rate. This metabolic response coincided with increased anaerobiosis, indicated by acidosis and the accumulation of lactate in their haemolymph. Changes in gas exchange during exposure and recovery to hypoxia in huhu are consistent with a strategy of metabolic depression. Larval anaerobiosis increased with the severity of hypoxic-temperature treatment. Knowledge of these metabolic responses at critical thresholds will be very useful in the development of commodity insect disinfestation methods.

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